Factors that affect the reproductive quality of honey bee queens and drones

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Honey bee reproductive quality

I. Factors that influence queen and drone reproductive health
II. The effects of grafting age on queen physiology and worker behavior
III. The effects of commonly used agro-chemicals on queen physiology and worker behavior
IV. The effects of commonly used agro-chemicals on drone reproductive health
V. Conclusions and future directions

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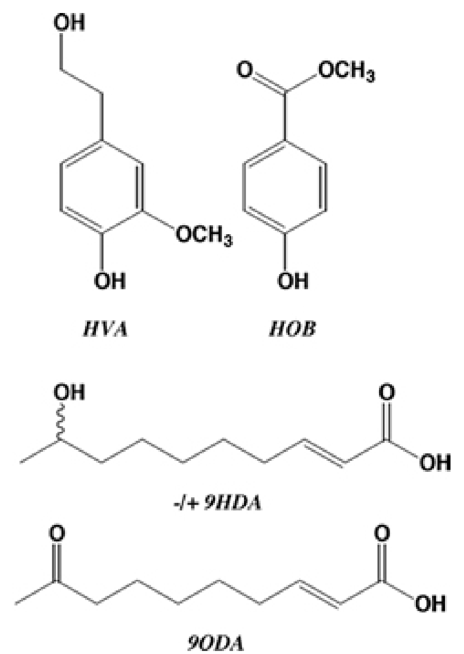
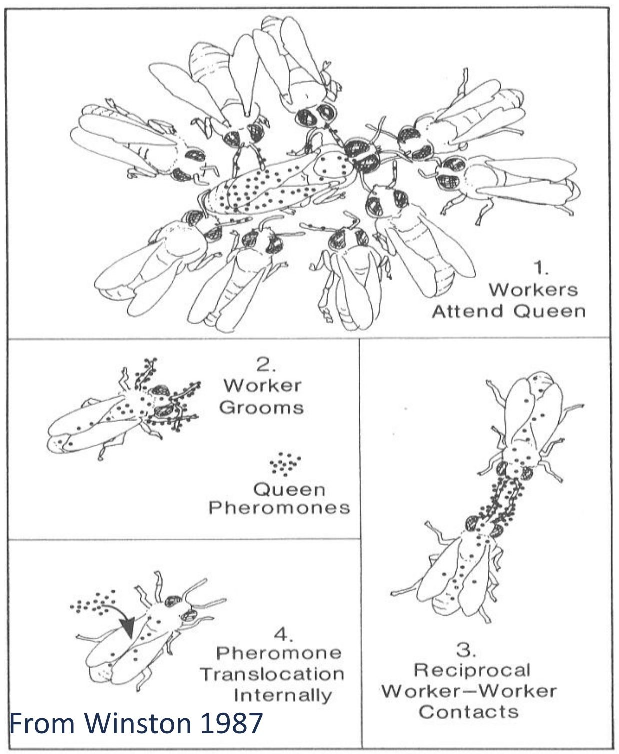
Honey bee reproductive division of labor

- Queens lay 1,500-2,000 eggs per day, while workers perform all other non-reproductive tasks
- Workers who care for the queen are called "queen's retinue"
- Queen pheromones elicit several physiological and behavioral responses in workers
- Workers are highly attracted to the queen's mandibular pheromones (QMP) which are unique in every queen

Queen Mandibular Pheromone (QMP)

- Produced by the mandibular glands
- A blend of 5 chemicals in different ratios (9-ODA, 9-HDA, HOB, HVA)
- Causes behavioral and physiological changes in colony members (workers)





From Winston 1987

- Workers groom and lick the queen, picking up her pheromones
- QMP are further transmitted among all colony members by contact and trophallaxis

QMP-modulated behaviors

- Elicits a "retinue response"
- Inhibits worker ovary activation
- Inhibits queen cell production
- Stimulates pollen foraging and brood rearing in small, newly founded colonies
- Increases nectar foraging
- Delays the age-at-onset of foraging
- Reduces JH production
- Attracts drones during mating

- QMP composition varies among queens based on reproductive state (Richards et al. 2007), development (Rangel et al. 2013), and pesticide exposure (Rangel et al. 2015, 2016)

Factors influencing reproductive quality

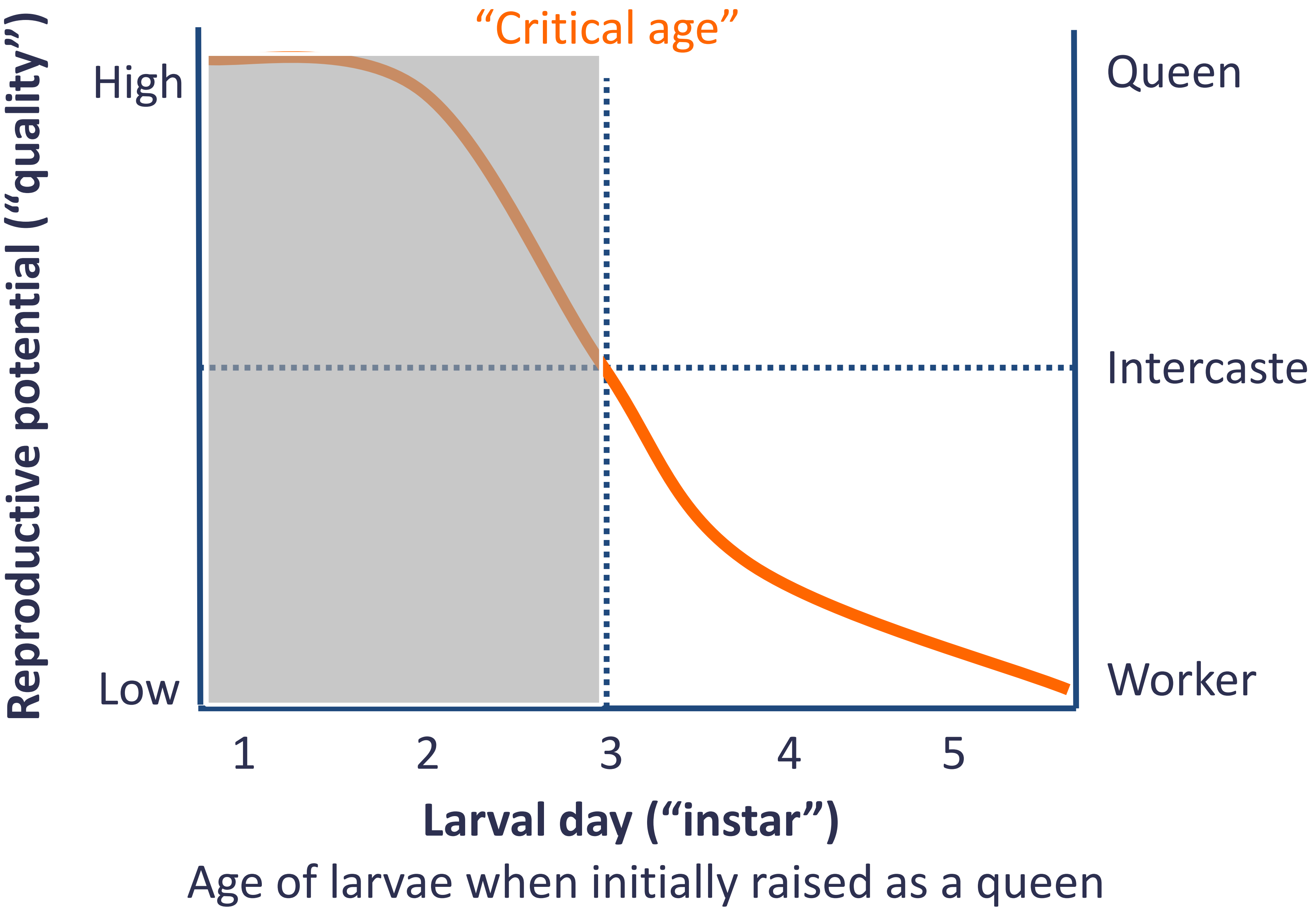
Biological factors

- Mating frequency
- Insemination volume
- Age of larva when initially chosen as a queen
- Pathogen types/levels
- Gut microbiota
- Sexual competitiveness
- Sperm counts
- Sperm viability, motility

Ecological factors

- Quality of nutrition
- Exposure to pesticides used in bee-pollinated crops
- Weather patterns
- Exposure to wax and food contaminated with in-hive chemicals during development

Variability in queen reproductive quality



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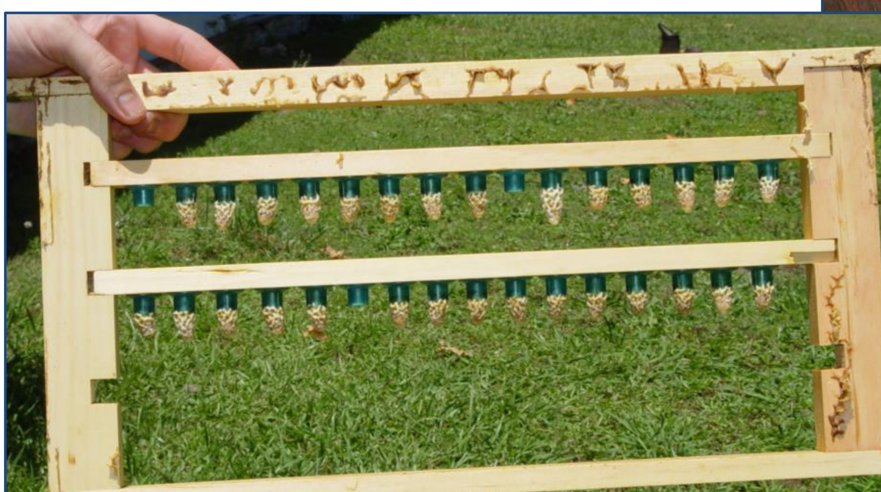
- Raised super-sister queens from 0-day-old and 2-day-old larvae

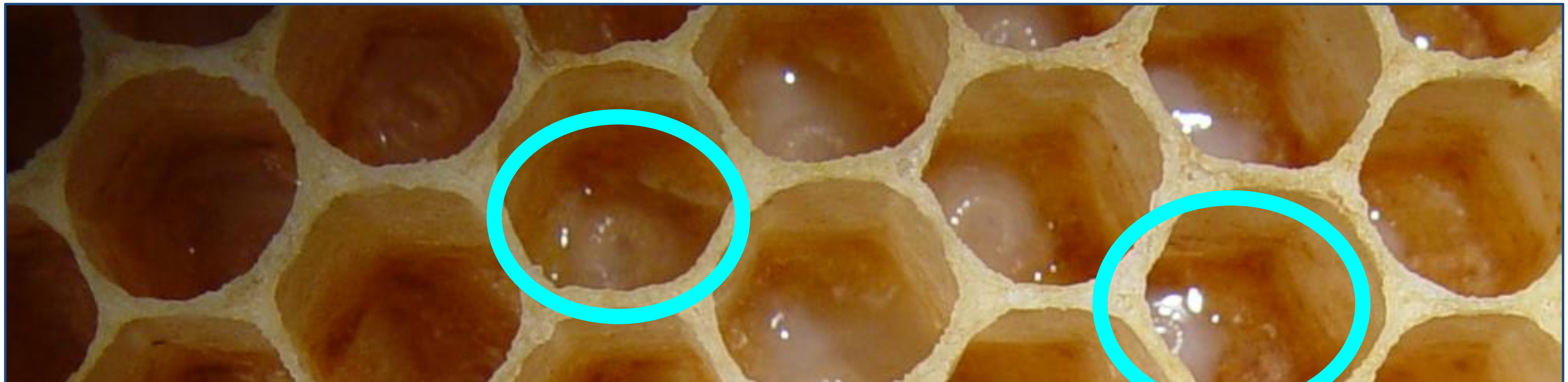
- Reared experimental queens by “grafting”



Differences in queen grafting age

Low-quality queens
2-day-old larvae

High-quality queens
0-day-old larvae



- Mature queen cells were placed into hives with ≈7,000 bees and allowed to mate naturally

QMP differences affect worker behavior

Are there differences in worker attraction to queens raised from young vs. old larvae?

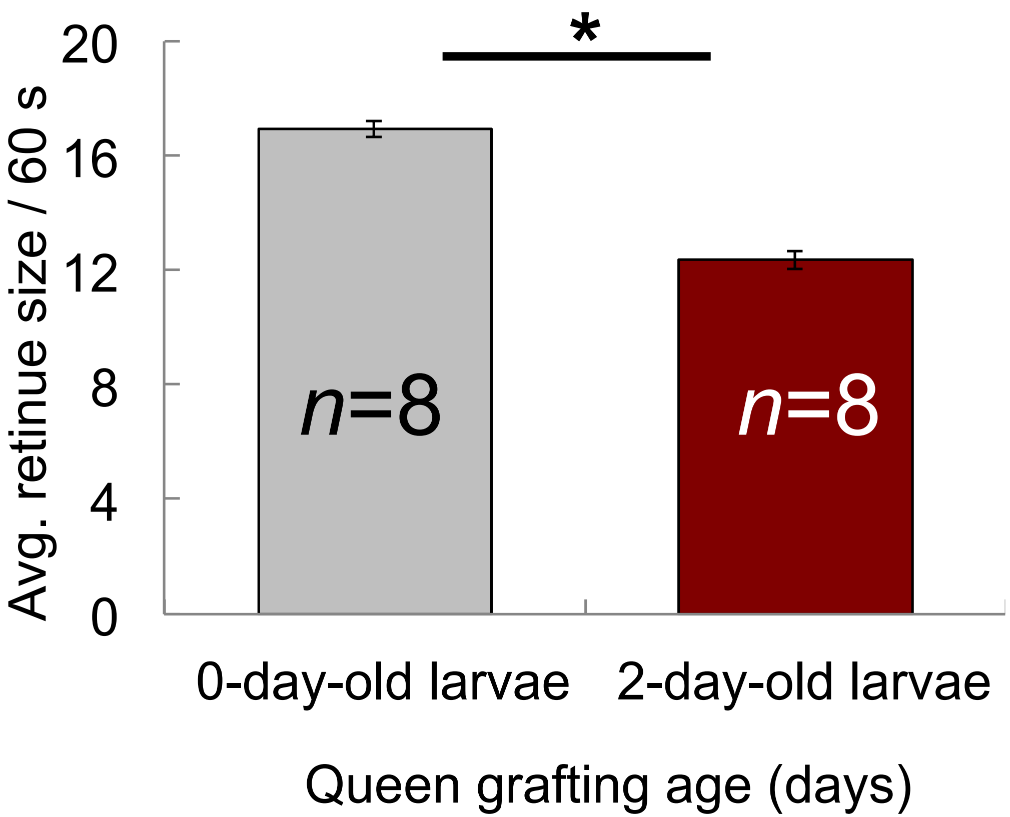
Differential queen rearing

"high" quality queens	"low" quality queens

- Placed mated experimental queens in pairs of observation hives with ≈6,000 bees

- Measured the number of workers in the queen's retinue for 60 sec during 5-min intervals, several days/pair

Variability in queen reproductive quality



- Workers were more attracted to high-quality queens than low-quality in retinue response bioassay

Queen quality affects colony phenotype

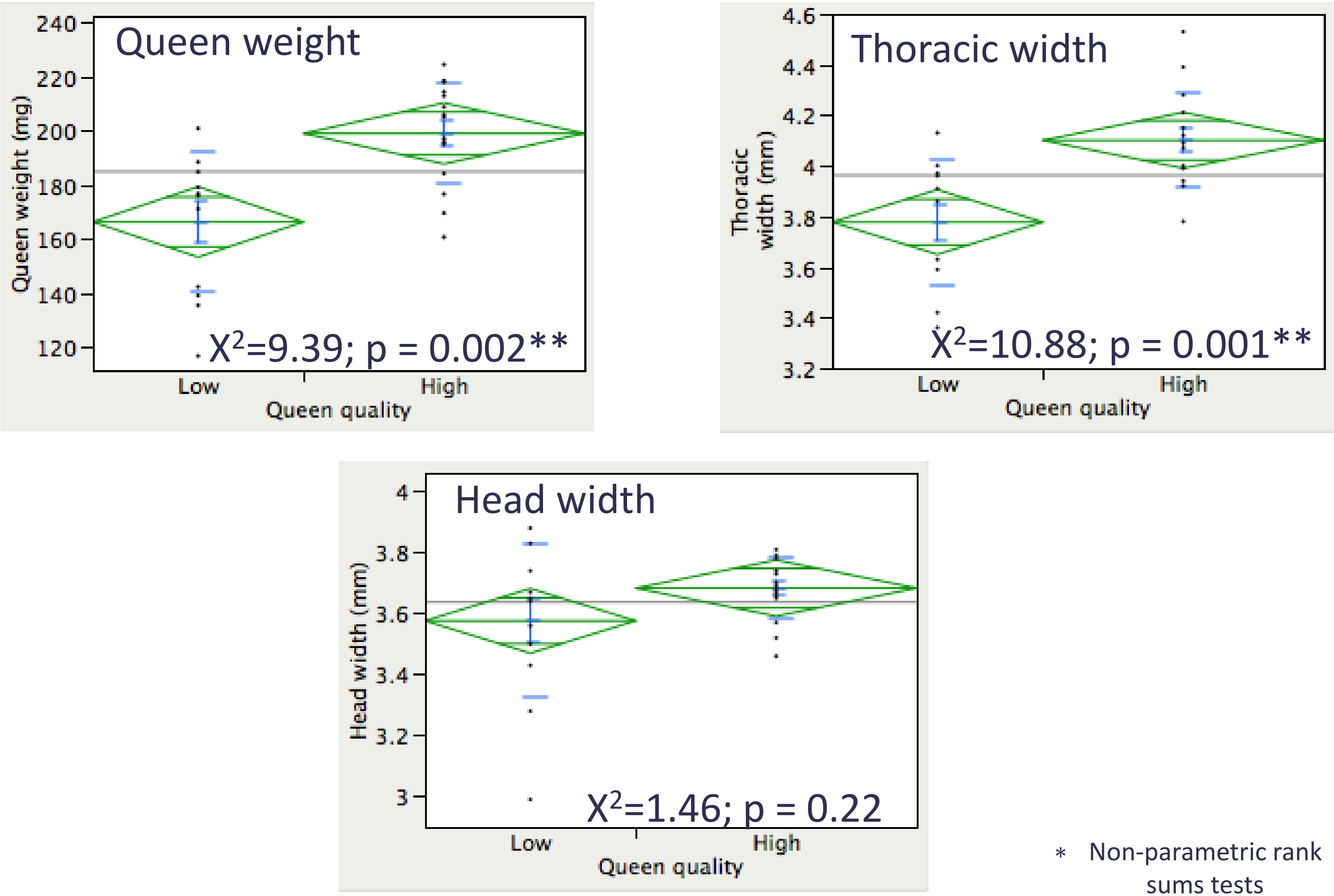
Experimental colonies

- 20 "high" quality queens
- 20 "low" quality queens

- Weight
- Thorax
- Head

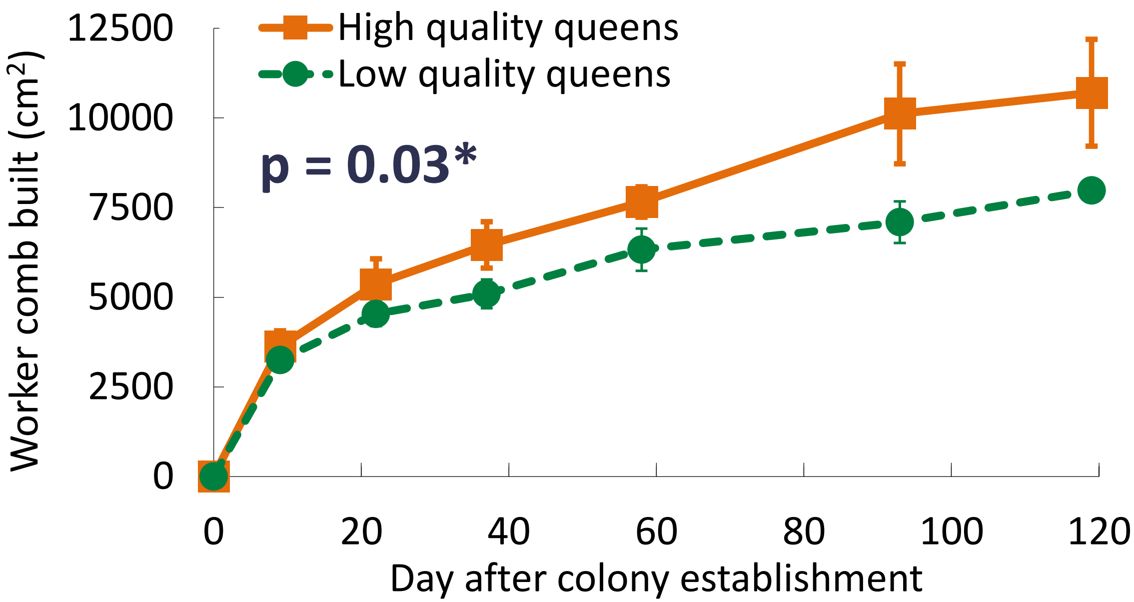
- Non-destructive measures taken monthly (in cm^2):
- Worker & drone comb
- Worker & drone brood
- Food stored
- Population size

Results: queen size differences



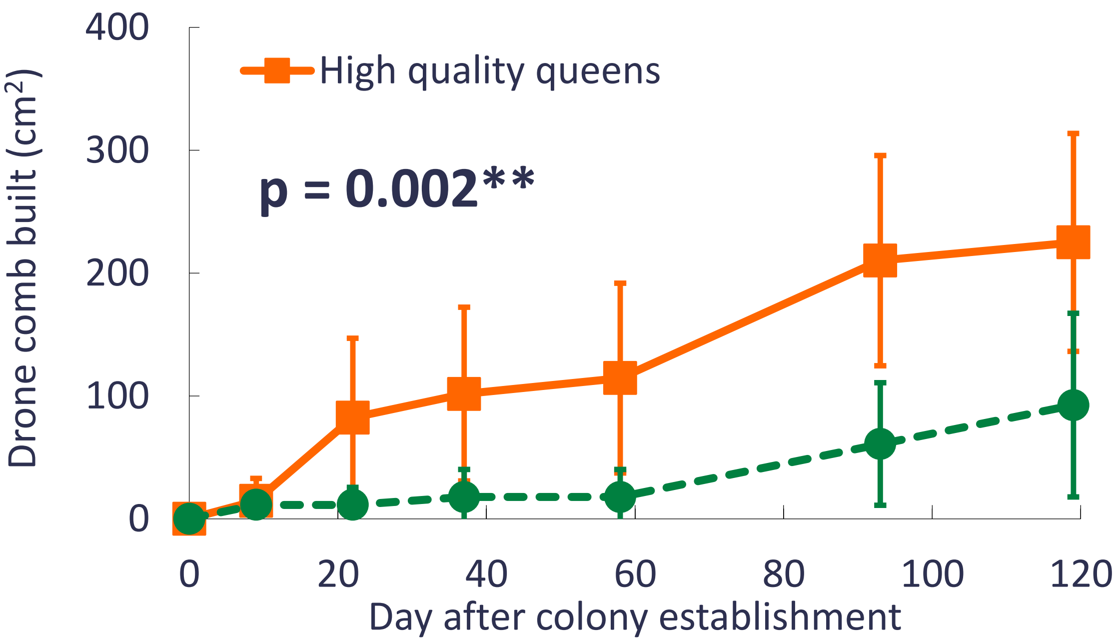
* Non-parametric rank sums tests

Results: amount of comb built



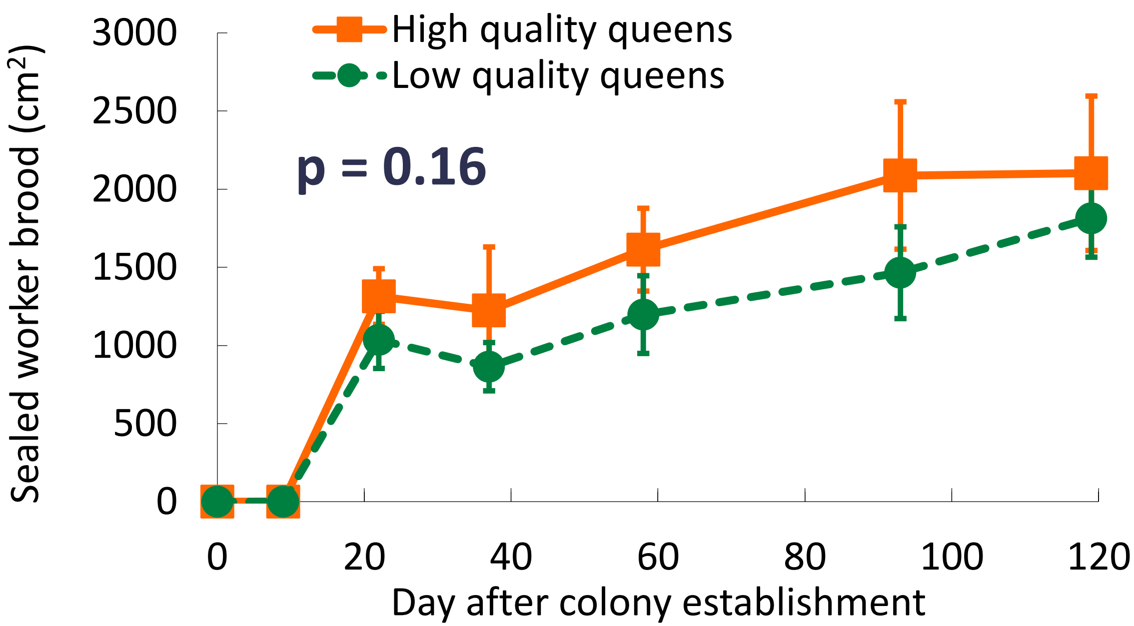
Worker comb

Drone comb



* Repeated measures ANOVA

Results: amount of brood reared

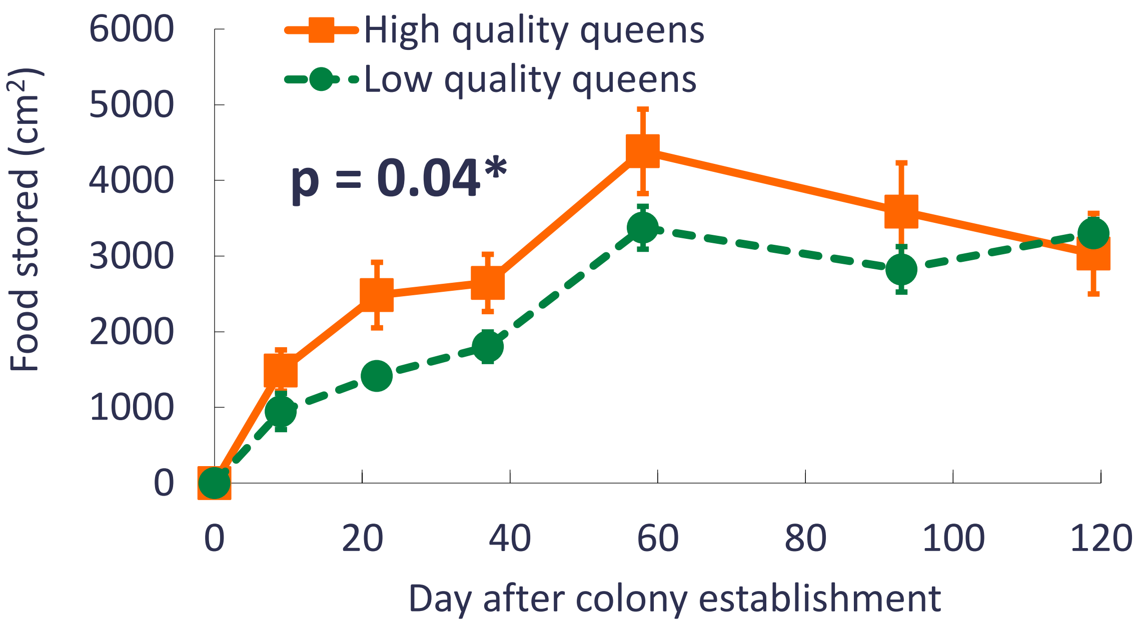


Worker brood

Drone brood

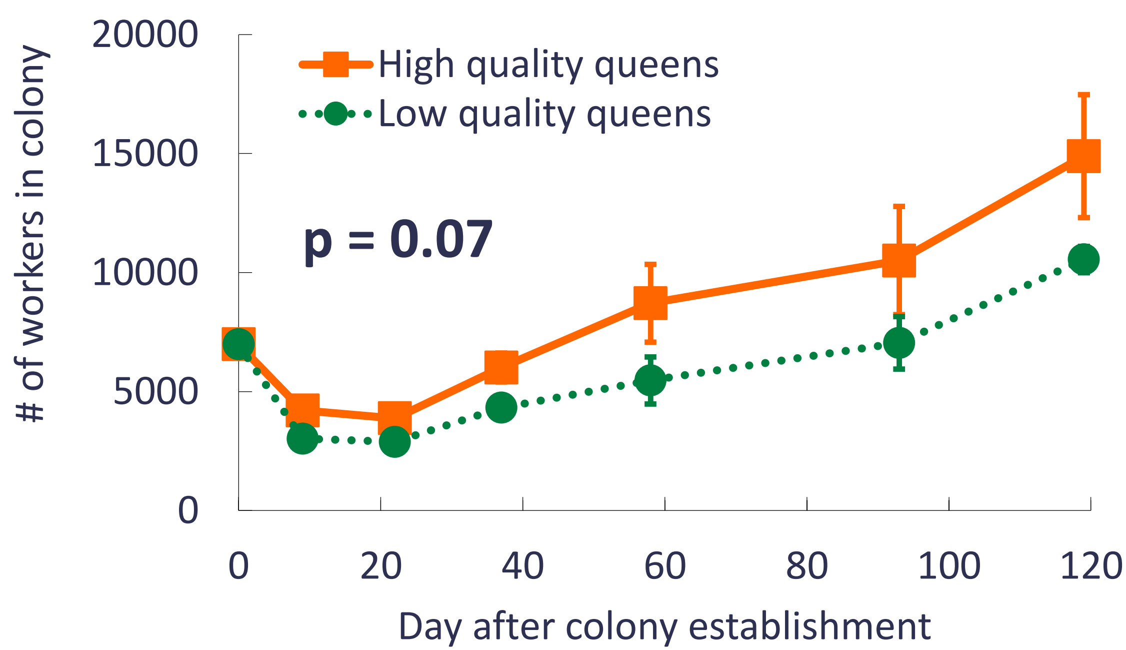
* Repeated measures ANOVA

Results: Food stored and adult population



Food stored

Population size



* Repeated measures ANOVA

Summary

- Queens raised from younger worker larvae exhibit higher reproductive quality
- "High-quality" queens exhibit differences in QMP chemical profile that is more attractive to workers
- New colonies headed by "high" quality queens had:
    - More worker and drone comb
    - Larger adult population
    - More food stored
    - More worker and drone brood
- Queen phenotype directly influences colony phenotype and fitness. QMP, among other queen-produced chemicals, seems to operate as an honest signal of queen quality

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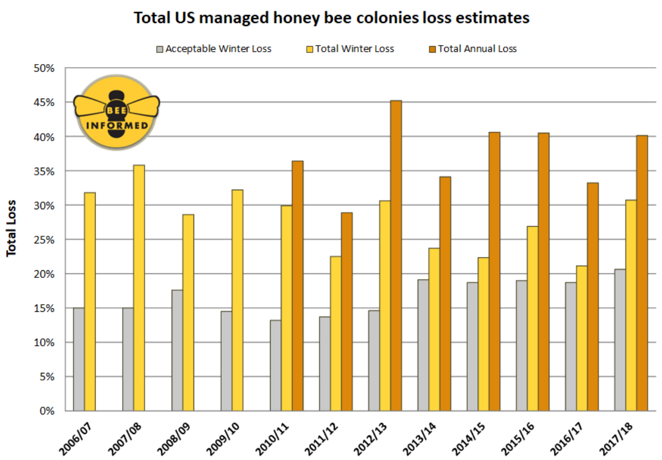
Beekeeper-reported colony losses



- Poor winter: 66% of colonies
- *Varroa* mites: 56% of colonies
- Pesticides: 66% of colonies
- Poor queens: 47% of colonies

Kulhanek *et al*. 2017

Commonly used miticides

Reduced abundance of mites across apiaries

Development of mites' resistance to miticides

Part of an IPM paradigm for honey bee health

Sublethal effects of miticides on honey bees

Non-target effects of pesticides on bees

- Honey bee colonies accumulate contaminants at levels detrimental to health & productivity (Konopacka *et al.* 1993, Haarmann *et al.* 2002, Mullin *et al.* 2010)

- Interaction between pesticides, mite stress and disease, contribute to colony declines (Cox-Foster et al. 2007, Frazier *et al.* 2008, Johnson *et al.* 2009, Wu *et al.* 2011)

- Fluvalinate and coumaphos negatively affect queen reproductive health (Haarmann *et al*. 2002, Rangel & Tarpy 2015)

- Lower ovariole weight and sperm viability, increased mortality

Experimental design

- Coated plastic queen cups with molten beeswax that was either miticide-free, or contaminated with a combination of fluvalinate and coumaphos

No miticides

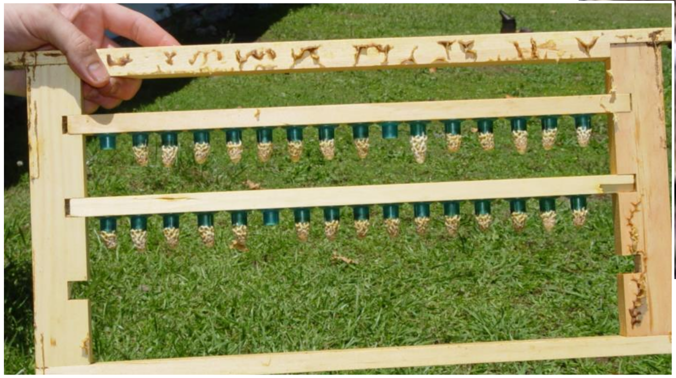
- Burt's bees miticide-free beeswax pellets

Fluvalinate + Coumaphos

- Maximum concentration of both miticides found in >250 wax samples (Mullin *et al.* 2010)
- 204 ppm of Fluvalinate
- 94 ppm of coumaphos

Queen rearing by grafting

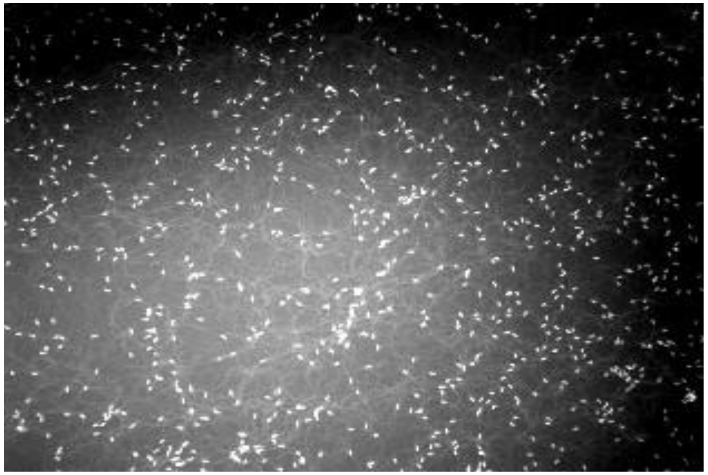
- Experimental queens were raised by "grafting"
- Collected sealed cells
- Mature queen cells were placed into new/empty "mating nucs" with ≈2,000 bees
- Emerged queens were used for measurements



Sperm viability analysis

Sperm counts

Sperm viability



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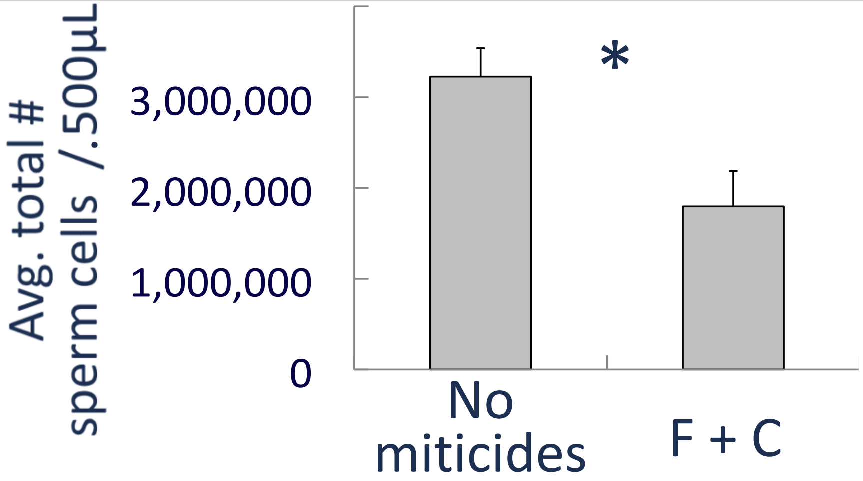
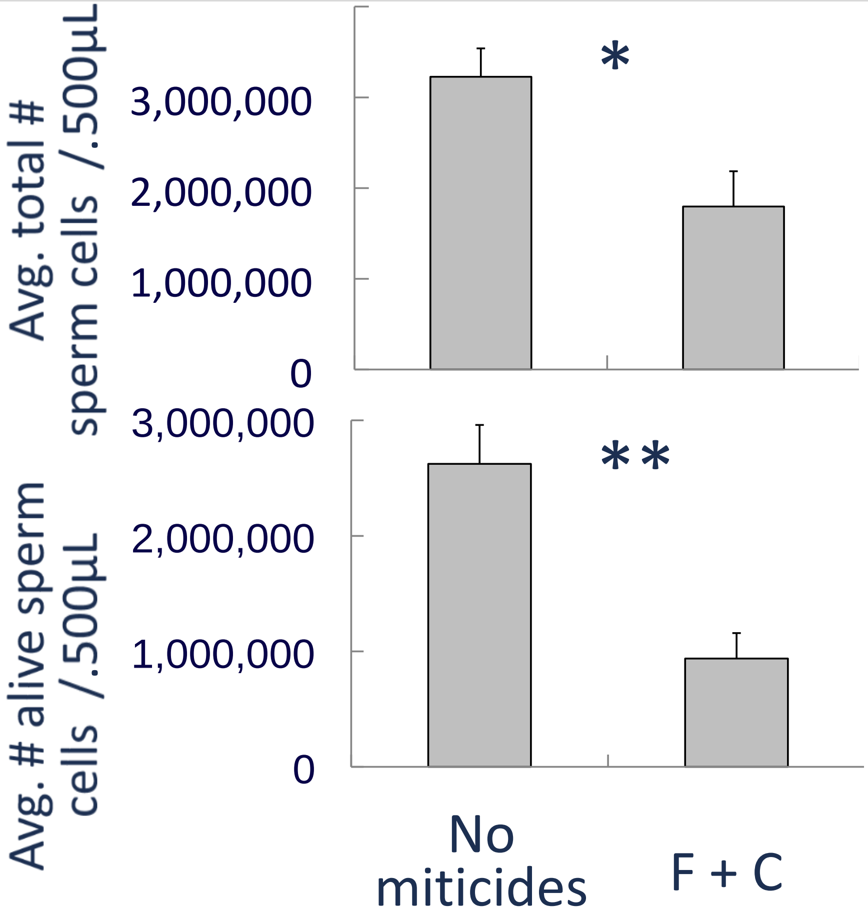


Avg. *total* # of sperm cells / 0.5 µL

F = 7.95; p = 0.01*

Sperm viability analysis



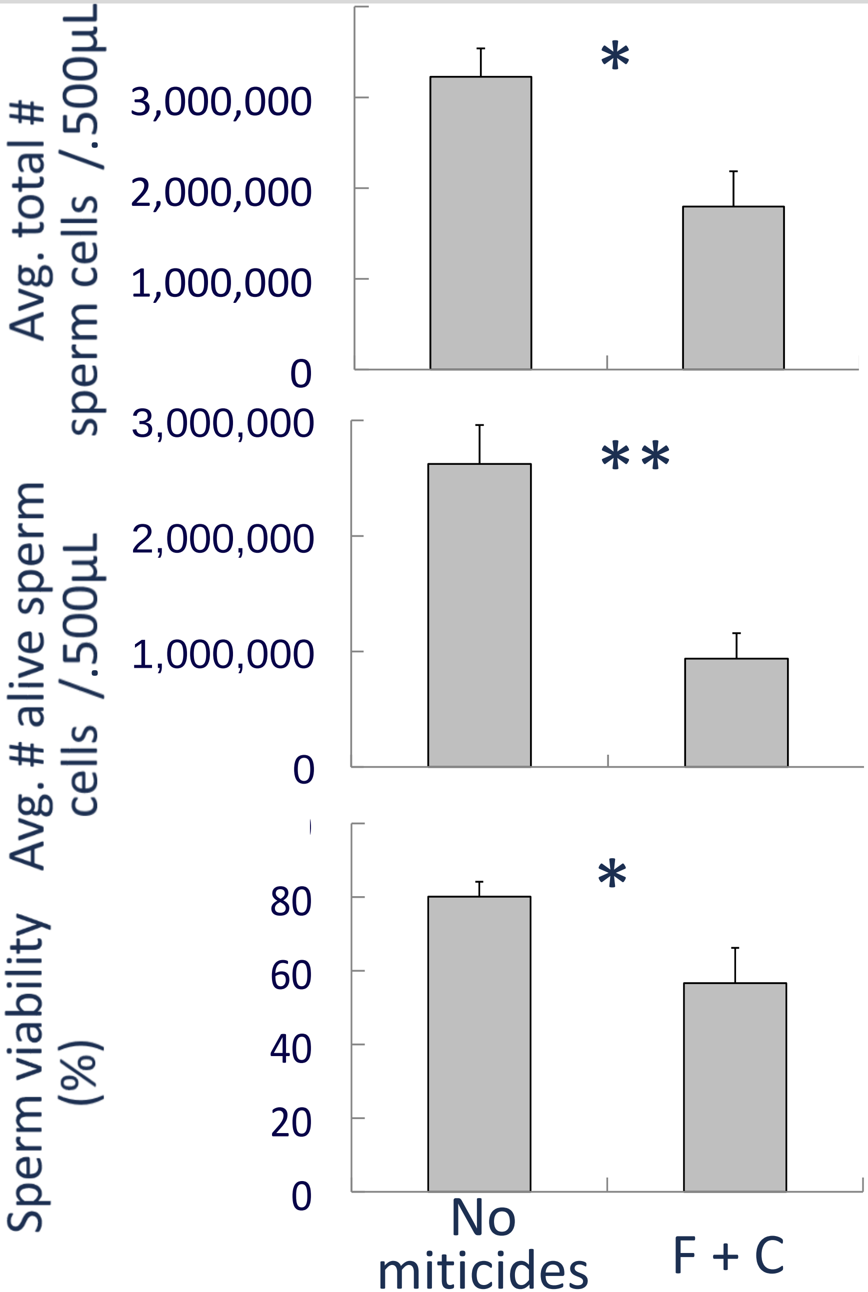
Avg. *total* # of sperm cells / 0.5 µL

F = 7.95; p = 0.01*

Avg. total # of *alive* sperm cells / 0.5 µL

F = 18.29; p = 0.0005**

Sperm viability analysis



Avg. *total* # of sperm cells / 0.5 µL

F = 7.95; p = 0.01*

Avg. total # of *alive* sperm cells / 0.5 µL

F = 18.29; p = 0.0005**

% sperm viability

F = 4.74; p = 0.04*

Liz Walsh, TAMU

Do queens reared in pesticide-contaminated beeswax have lower reproductive quality than queens reared in pesticide-free beeswax?

Effects of miticides on queens

Compared to queens reared in pesticide-free beeswax, queens reared in beeswax contaminated with pesticides will exhibit:

Smaller "retinues"

Photo: Kathy Garvey

Lower egg-laying rate

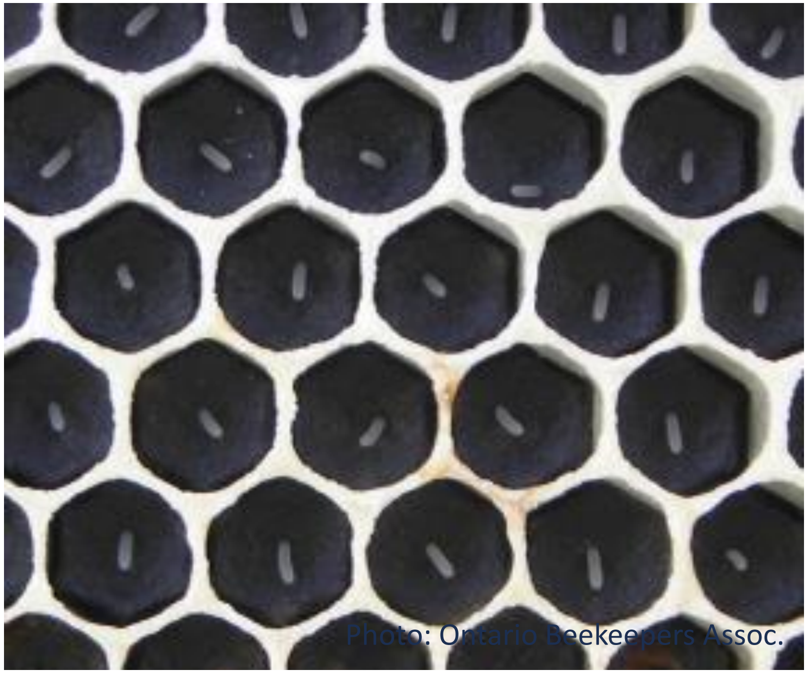


Photo: Ontario Beekeepers Assoc.

Agro-chemical contamination in beeswax

- Lipophilic beeswax in most commercial hives is widely contaminated with several agro-chemicals (Mullin *et al*. 2010)
  - Pesticides/fungicides

Table 1. Summary of pesticide detections in wax From Mullin et al. 2010

Wax Pesticide*	Class#	Detects	Samples Analyzed	%	Detections (ppb) High	Low
Fluvalinate	PYR	254	259	98.1	204000.0	2.0
Coumaphos	OP	254	259	98.1	91900.0	1.0
Coumaphos oxon	OP	187	208	89.9	1300.0	1.3
Chlorpyrifos	OP	163	258	63.2	890.0	1.0
Chlorothalonil	FUNG	127	258	49.2	53700.0	1.0
DMPF (amitraz)	FORM	107	177	60.5	43000.0	9.2

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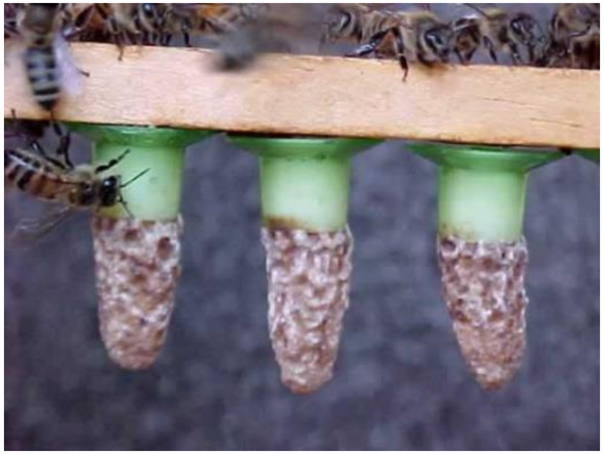
Queen cup preparation

Step 1: Coat each cup w/200 mg of molten wax

Control — Pesticide-free wax

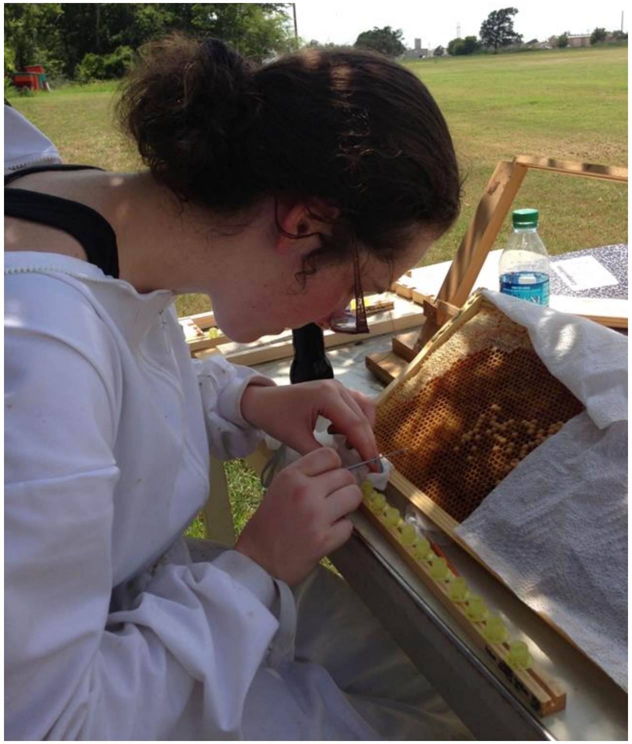
Experimental

- 20.4 ppm fluvalinate
  9.4 ppm coumaphos
- 4.3 ppm amitraz
- 0.1 ppm chlorpyrifos
  5.4 ppm chlorothalonil



*Field-relevant concentrations based on Mullin *et al.* (2010)

Queen rearing and observation hives



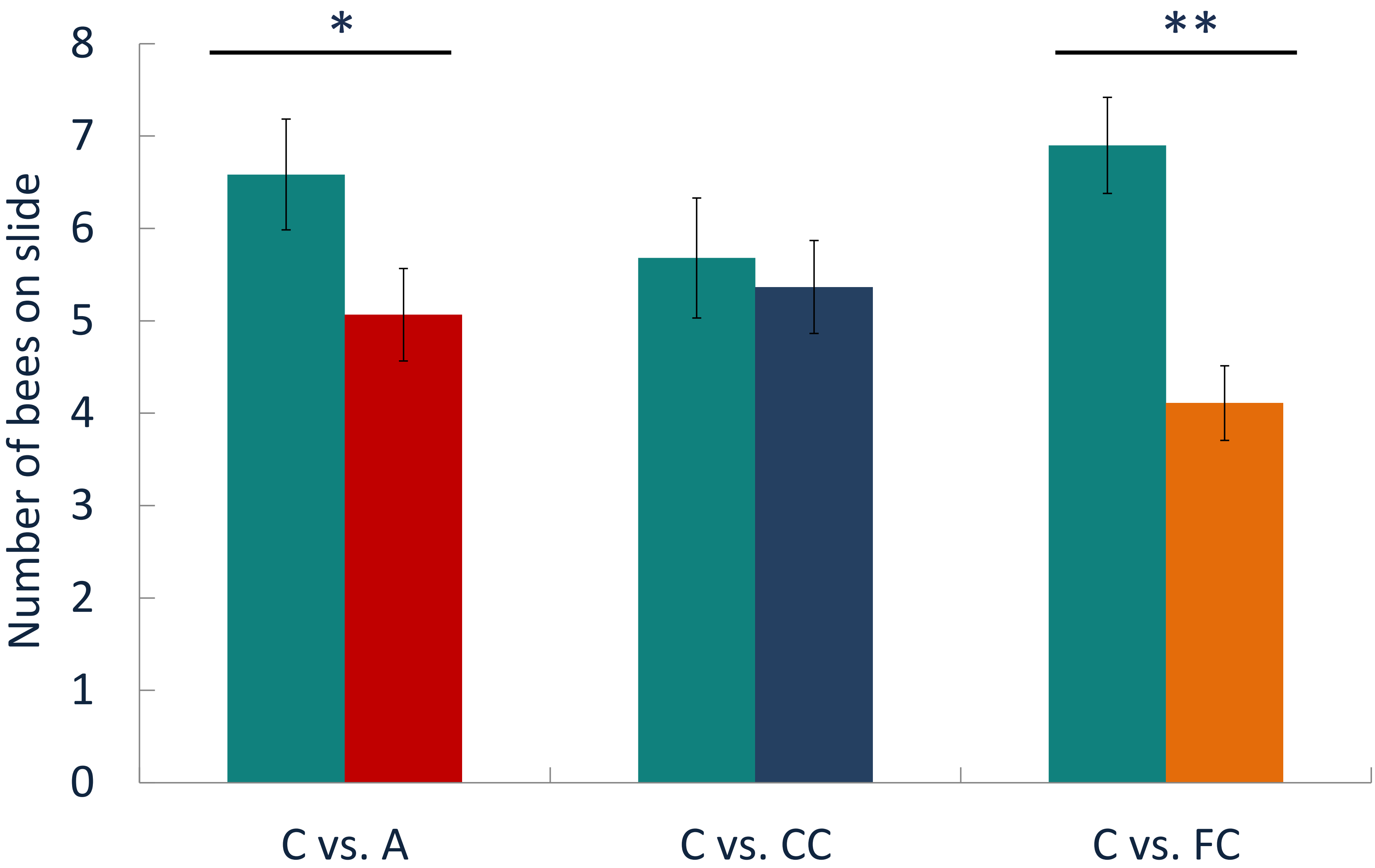
- Grafted one-day-old larvae
- Introduced queen cells to queenless cell builders
- Allowed queens to mature and mate naturally

- Each mated queen introduced to a randomly chosen queenless observation colony

Choice tests, behavioral assays

- Collected emerging workers
- Control and experimental gland extracts on two slides
- Choice test of caged 5-day-old workers with 0.5 Qeq (Richards *et al.* 2017, Niño *et al*. 2013, Rangel *et al.* 2016)
- Measured retinue size and egg laying rate
  - # workers or eggs laid/5-min
  - Point sampled throughout day
  - Min. of 50 5-min observations

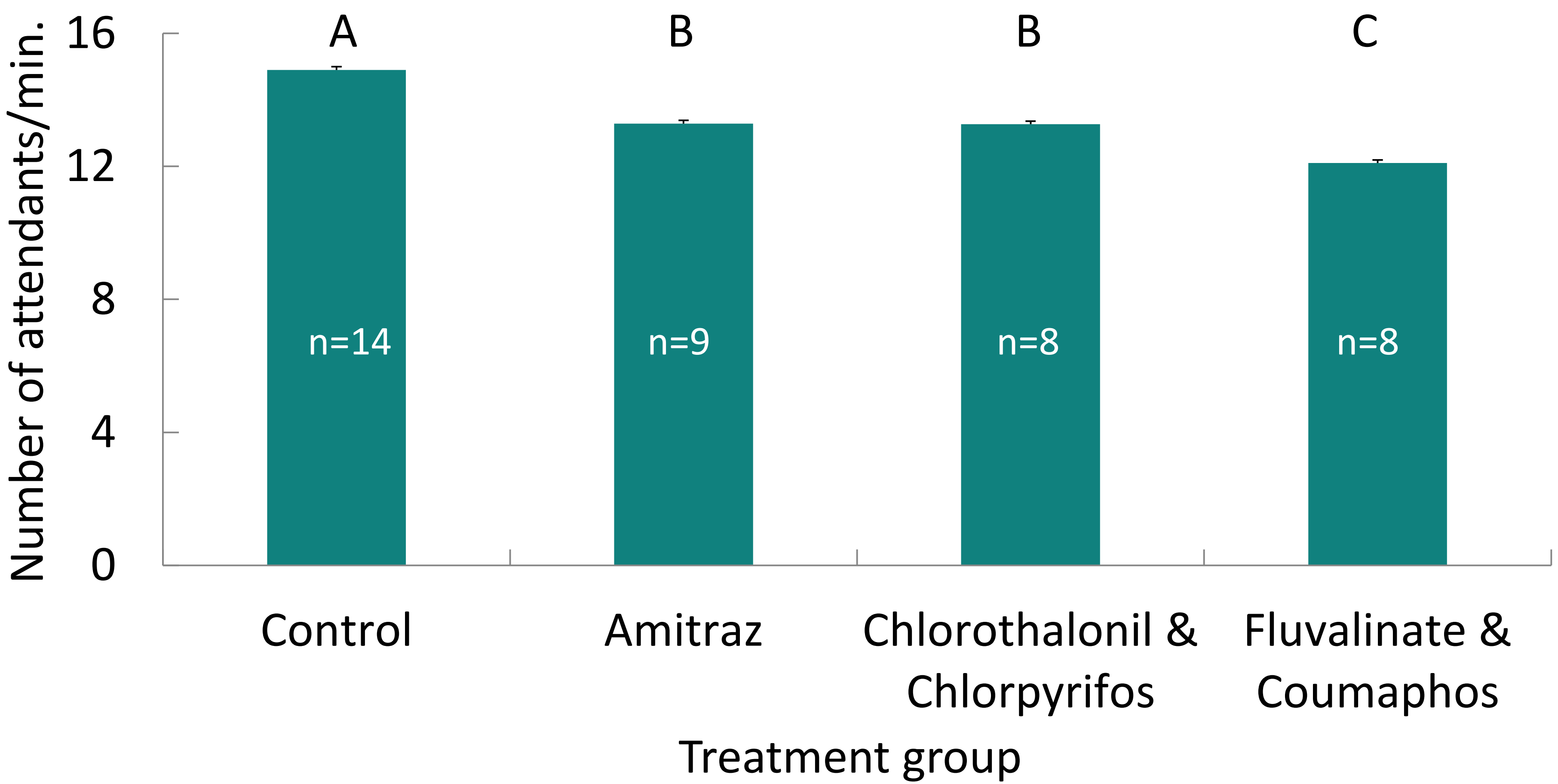
Significant differences in choice tests



Bioassay of worker choice test (t-test, t=1.966 , N=6/comparison; P=<0.05*; P=<0.005**)

Results: differences in retinue size

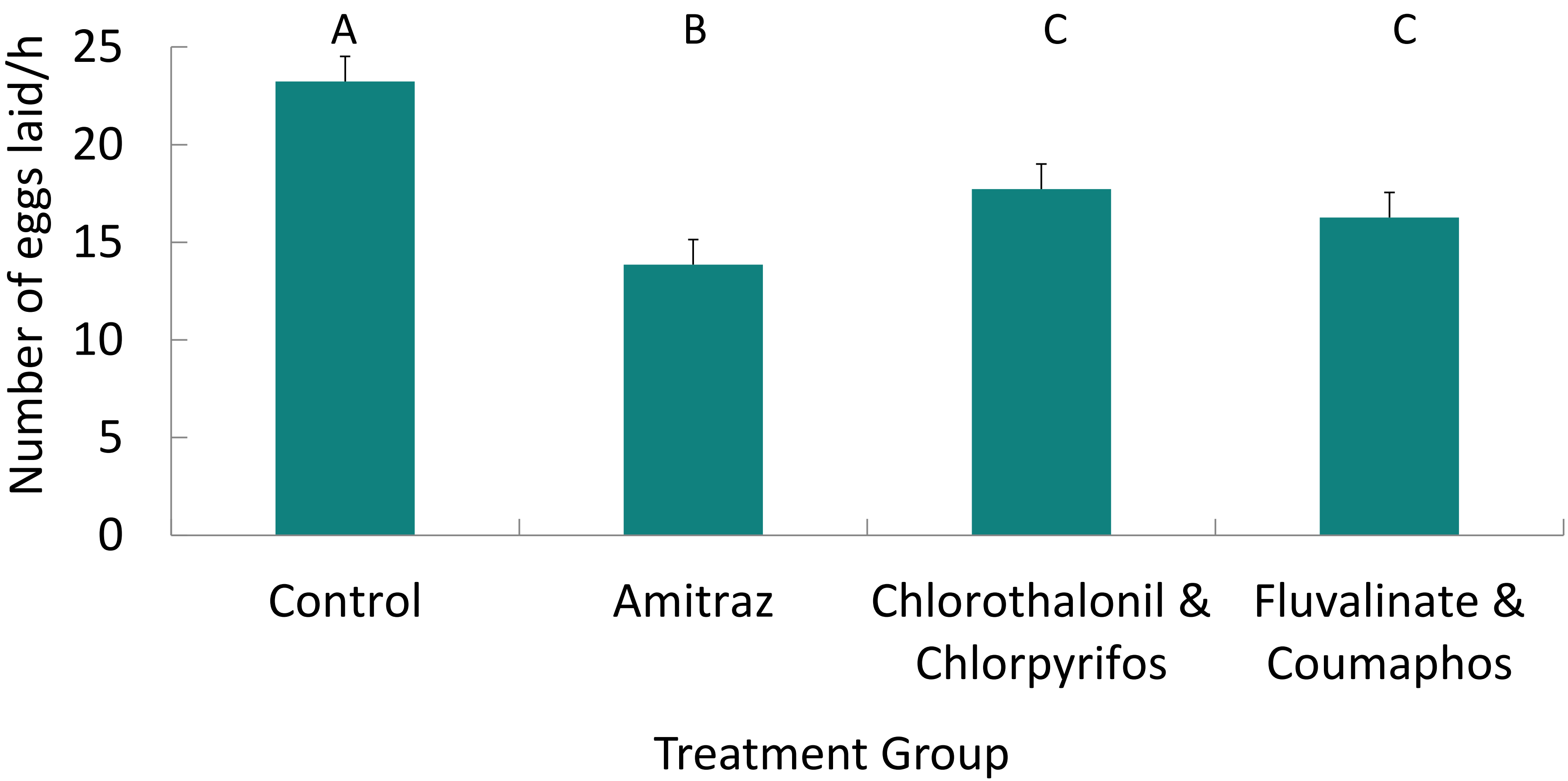
Significantly smaller worker retinue in colonies headed by queens reared in pesticide-laden wax



Overall average number of workers in retinue per treatment. (ANOVA, $^*P < 0.0001$).

Results: differences in egg-laying rates

Significantly fewer eggs laid per hour in colonies headed by queens reared in pesticide-laden wax



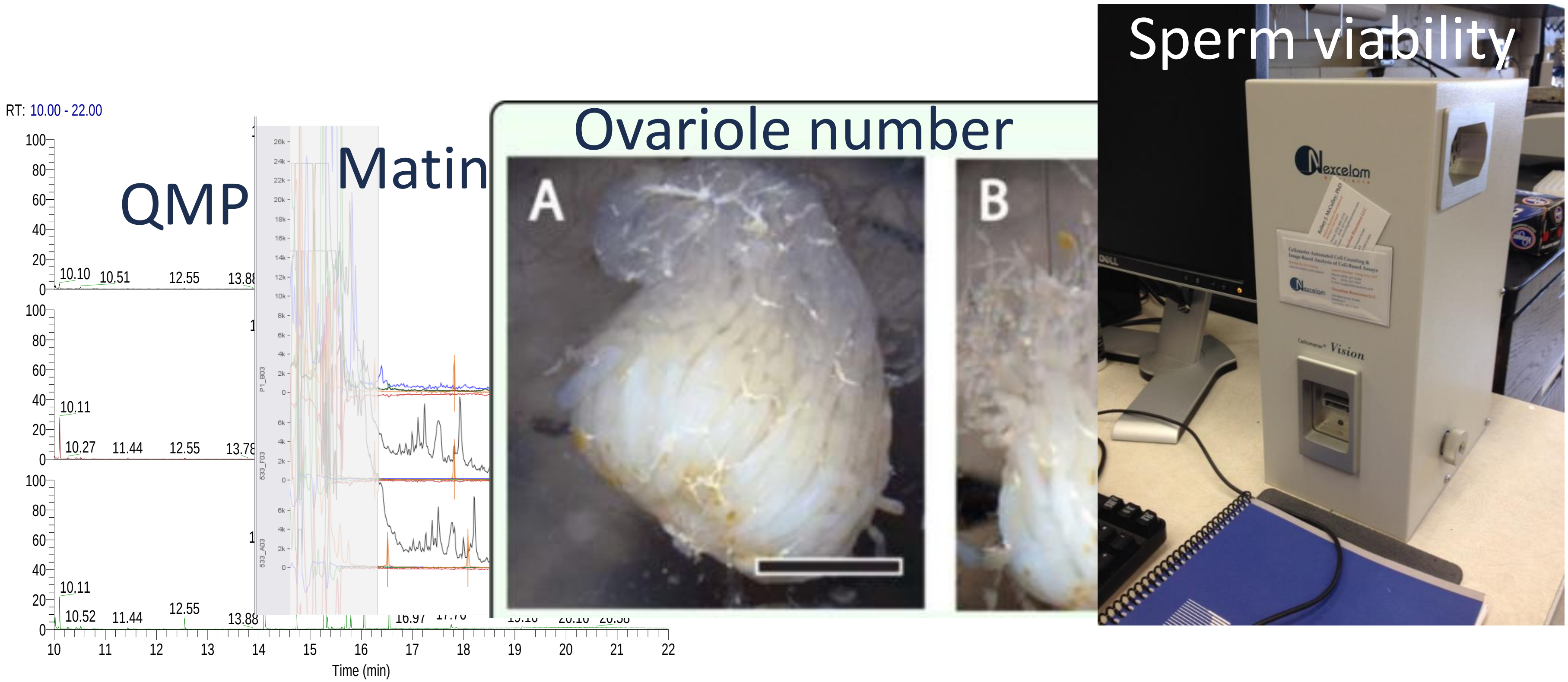
Control mean: 23.32 ± 0.02; Amitraz mean: 13.85 ± 0.01; C+C mean: 17.72 ± 0.02; F+C mean: 16.26 ± 0.01; Tukey-Kramer, $P<0.0001$.

Summary

- Synergistic effects of miticide and agro-chemical contamination of wax on queen reproductive health
- Compared to those raised in pesticide-free wax, queens raised in pesticide-laden wax had:
  - Lower total number of sperm cells
  - Lower avg. number of alive sperm cells
  - Lower sperm viability
  - Smaller worker retinues
  - Lower egg-laying rate

Ongoing work

- Assess the long-term effects of miticide contamination of the queen-rearing wax environment on:



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Adrian Fisher II, ASU

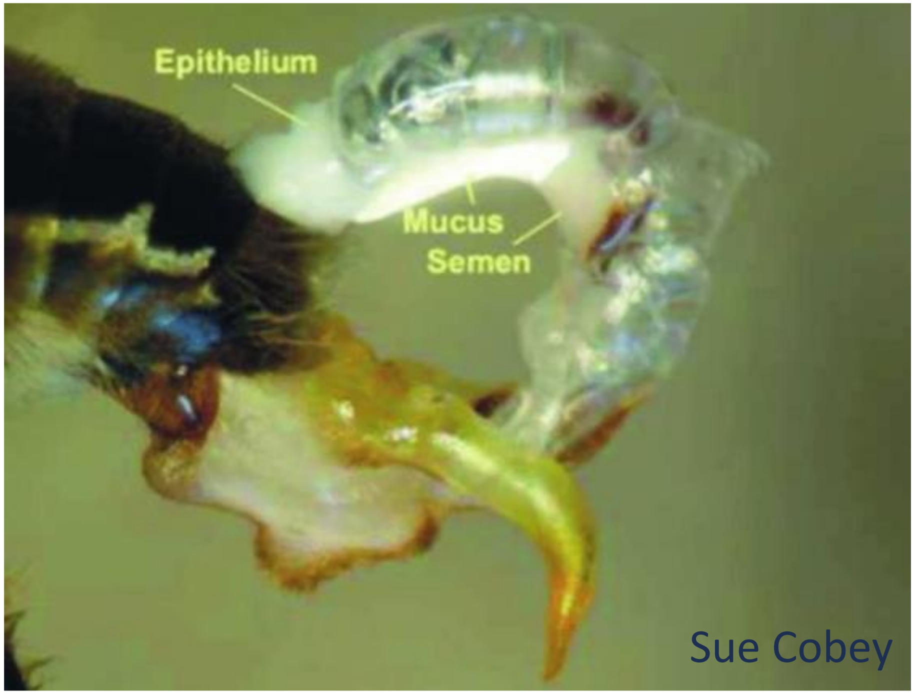
Do drones reared in pesticide-contaminated beeswax have lower reproductive quality than queens reared in pesticide-free beeswax?

Drone biology

- A colony produces thousands of drones during the swarming season only if environmental conditions allow it, and if the colony is large enough
- All the sperm are formed before a drone's emergence from the cell, but the sperm needs to migrate into the seminal vesicles before the drone can mate (>10 days post-emergence)

Drone biology

- Mature drones have ≈ 10 million spermatozoa in seminal vesicle, about 1 µL of semen
- But a drone provides <1 million spermatozoa to a queen's spermatheca
- A well-filled spermatheca contains ≈ 5+ million spermatozoa



Pesticide concentrations used

Experimental group	Pesticides used	Concentrations used
Control	No pesticides	0
Treatment 1	Fluvalinate Coumaphos	20.4 mg/100 mL acetone 9.2 mg/100 mL acetone
Treatment 2	Chlorothalonil Chlorpyrifos	5.4 mg/100 mL acetone 0.1 mg/100 mL acetone
Treatment 3	Amitraz	4.3 mg/100 mL acetone

- Wax with pesticide diluted in acetone and sprayed on coated frames, put into hives (Johnson et al. 2013)

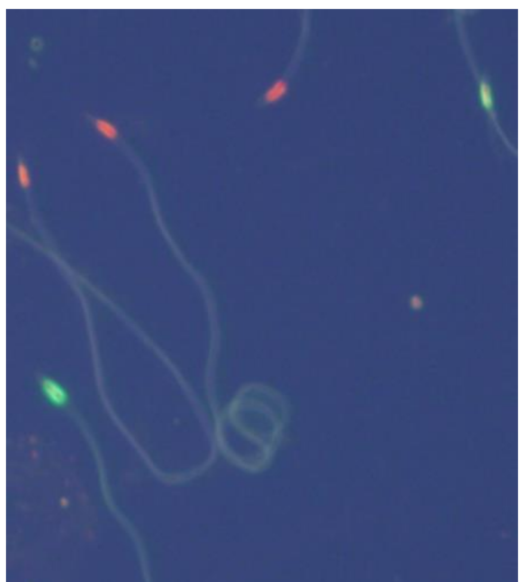
Drone rearing

- Sealed drone brood incubated at 34^{o}C

- Emerged drones labeled, placed back into source colony
- Waited 16+ days and collected adults once sexually mature

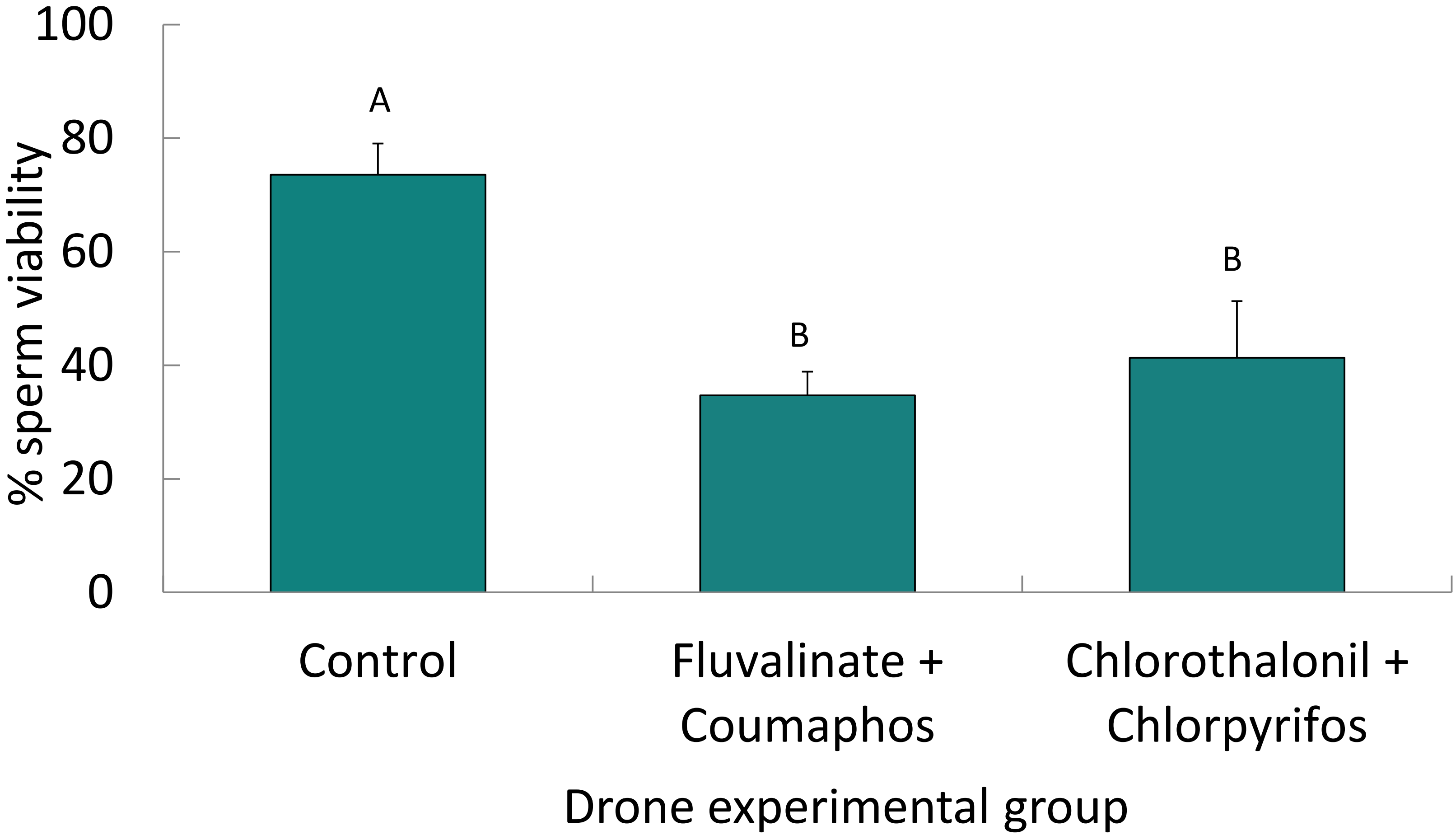
Semen collection and viability assessment

- Collected semen samples from sexually mature (18+ days) and immature drones (10 days) (Collins and Donoghue 1999)
- Semen samples stained with Sybr-14 & Propidium Iodide



Mature drone sperm viability

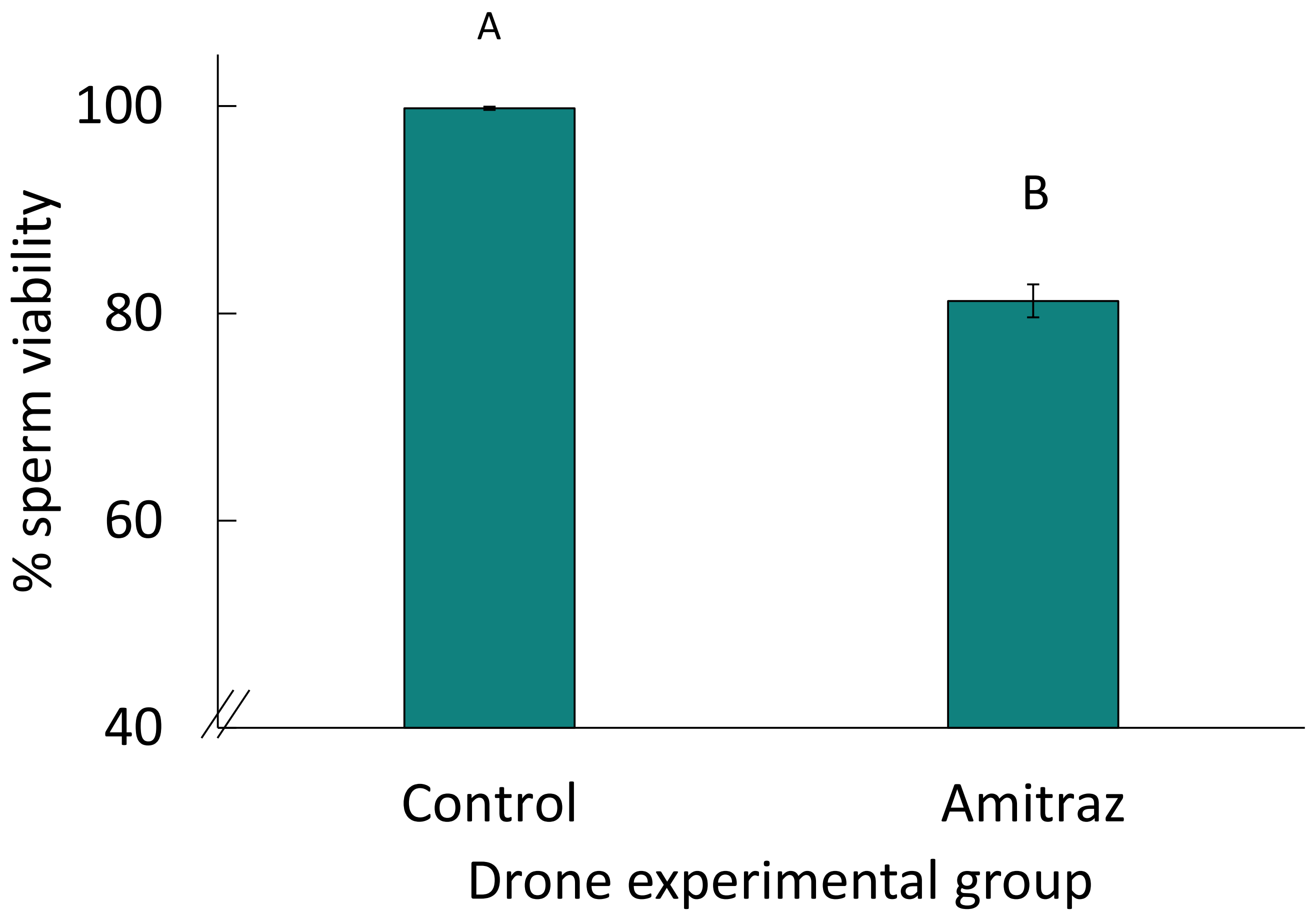
Significantly lower sperm viability in mature drones reared in pesticide-laden wax



- Control sperm viability significantly higher than F+C treatment (t-ratio = 5.12, df = 21, $P < 0.0001$) and C+C treatment (t-ratio = 3.39, df = 9, $P = 0.009$)
- No significant difference between F+C and C+C (t-ratio = 0.66, df = 21, $P = 0.51$)

Mature drone sperm viability

Significantly lower sperm viability in mature drones reared in amitraz-laden wax



Control sperm viability significantly higher than amitraz treatment (t-ratio = 11.5, df = 13, $P < 0.0001$)

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Conclusions and ongoing work

- Miticides and agro-chemicals negatively affect drone spermatozoa viability
  - How does nutrition affect drone quality?
  - How does spermatozoa quality affect mated queen phenotype and supersedure rates?
- Recommend alternative *Varroa* control practices
  - Assess drone quality when conducting breeding programs
  - Replace old combs
  - Reduce or eliminate with non-chemical IPM

Thank you! Any questions?

Texas A&M University Apiculture
@TAMUhoneybeelab

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Aerial Application Technology

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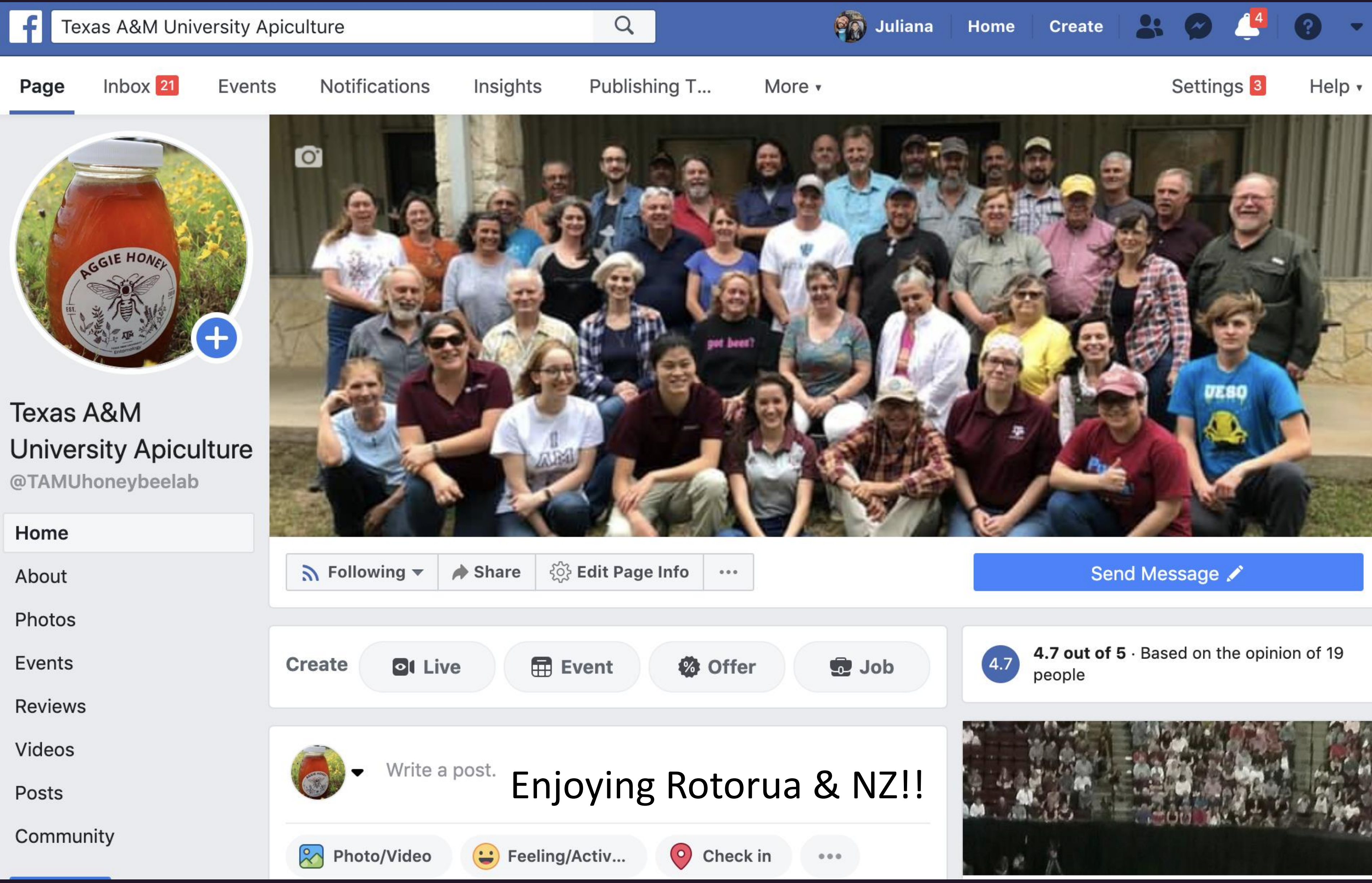

Sweet Safari

TEXAS A&M UNIVERSITY

Department of Entomology

Thank you! Any questions?

jrangel@tamu.edu

Bee lab's website: http://honeybeelab.tamu.edu

Texas A&M Honey Bee Lab

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Welcome to the Texas A&M University Honey Bee Research Program's website!

The Texas A&M University Honey Bee Research Program, led by Dr. Juliana Rangel, Associate Professor of Apiculture in the Department of Entomology, focuses on basic and applied research, as well as education and outreach regarding honey bee biology and management. Our research interests revolve around the reproductive biology of honey bee queens and drones, the behavioral ecology and population genetics of feral and managed honey bee colonies, as well as pollination and beekeeping practices.

Please follow us on Facebook for the latest updates:https://www.facebook.com/TAMUhoneybeelab

Latest News:

5th Annual "*ART OF QUEEN REARING*" Workshop Saturday, 18 May 2019

Janice and John G. Thomas Honey Bee Facility, College Station, TX Head Instructor: Dr. Juliana Rangel
Special Guest: Sue Cobey

Co-Instructors: ET Ash, Pierre Lau, Alex Payne, Liz Walsh, Taylor Reams

Alex Payne, TAMU

How does contamination of wax foundation affect the growth of new honey bee colonies?

Experimental colony set up

- 30 nucleus colonies (nucs) set up
- Each nuc had 5 uncoated Plasticell frames
- Alternating full and partial foundation

Control group (Cont)	Treatment Group 1 (F+C)	Treatment Group 2 (C+C)	Treatment Group 3 (A)
Pesticide-free acetone	Fluvalinate: 20.4 ppm Coumaphos: 9.2 ppm	Chlorothalonil: 5.4 ppm Chlorpyrifos: 0.9 ppm	Amitraz: 4.3 ppm

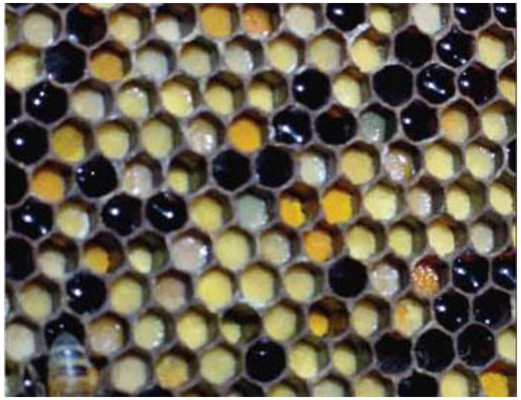
(Mullin et al. 2010)

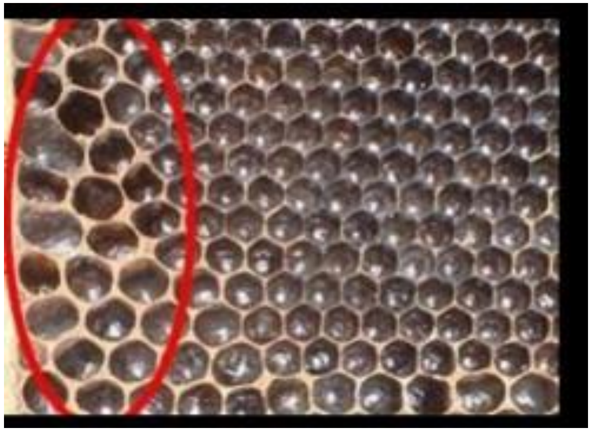
- Frames coated in pesticide-free wax, then sprayed with treatment in acetone

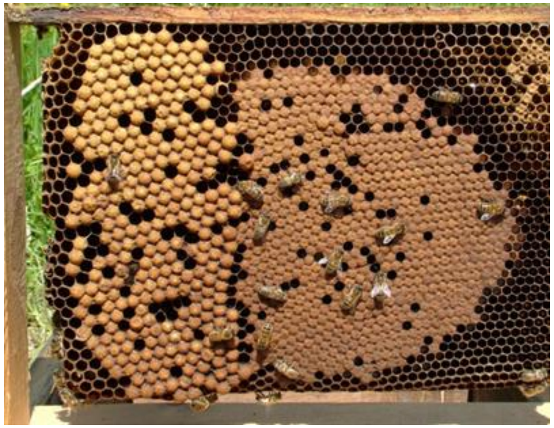
Photo: P Patch

Colony growth measurements

- Experiment conducted from May-October 2017
- Parameters measured to determine colony growth:
  - Comb built (worker and drone)
  - Capped brood (worker and drone)
  - Food stored (honey, nectar, pollen)
  - Population of each colony
  -



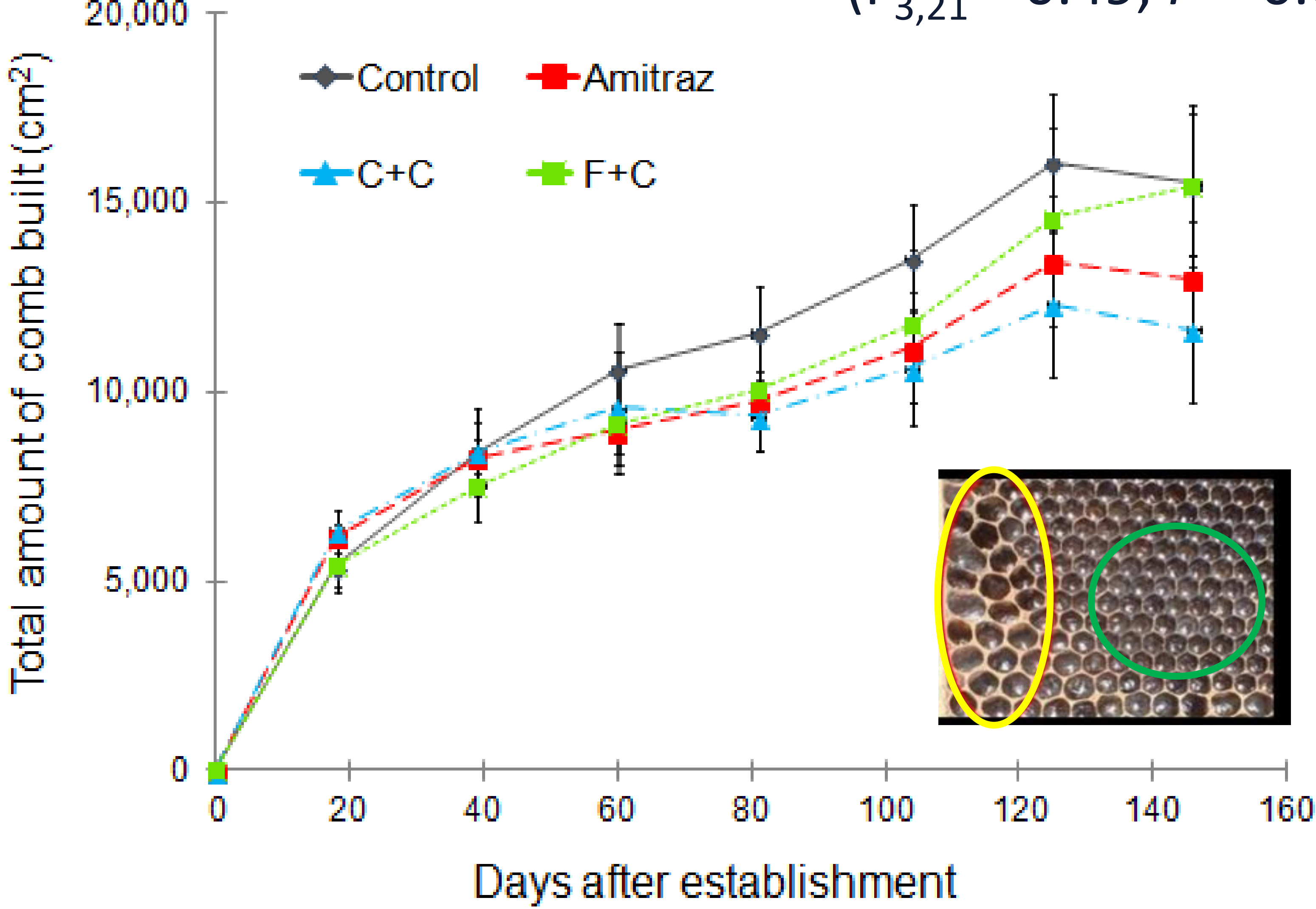




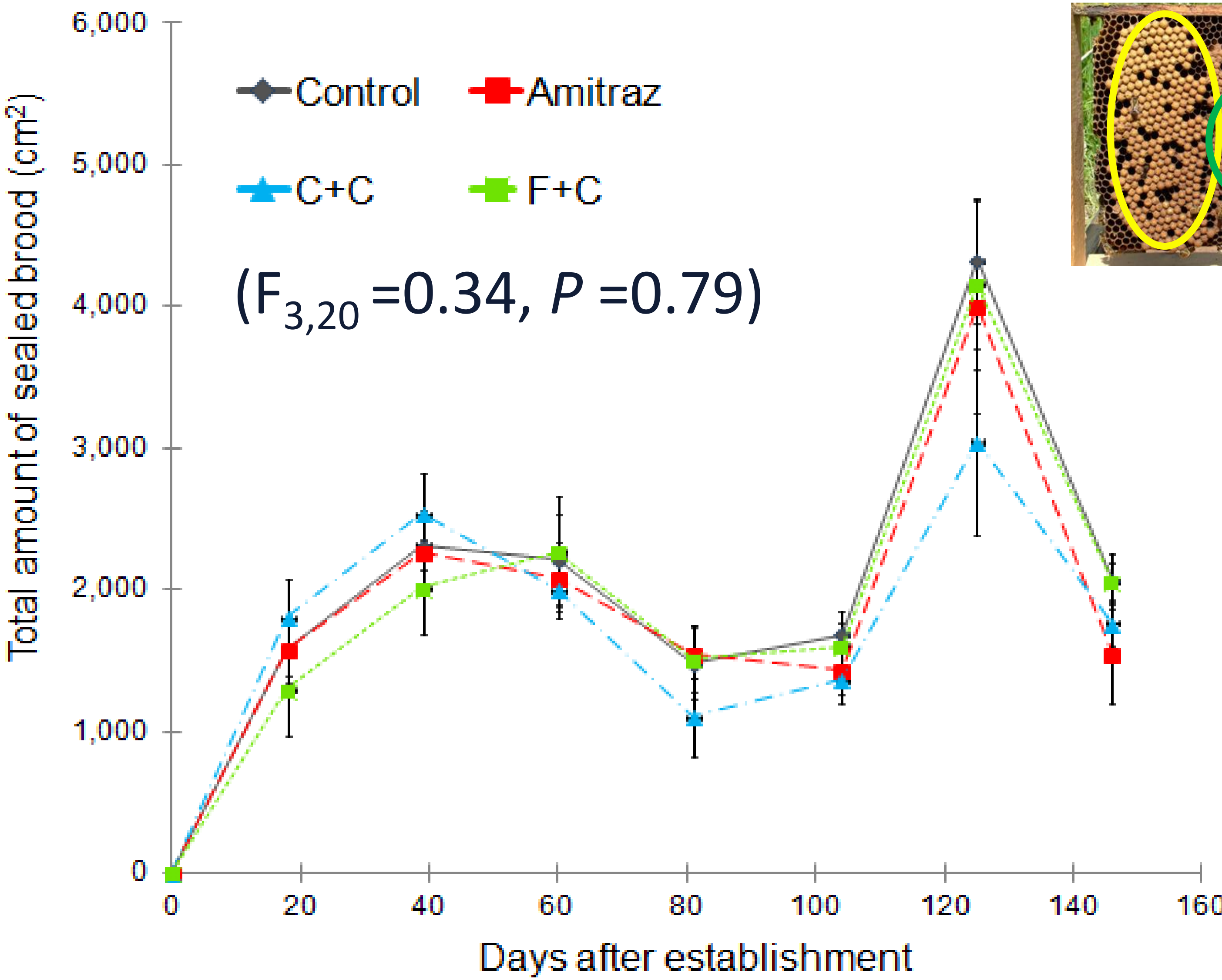
Gridded wooden frame (in^2), then extrapolated to cm^2

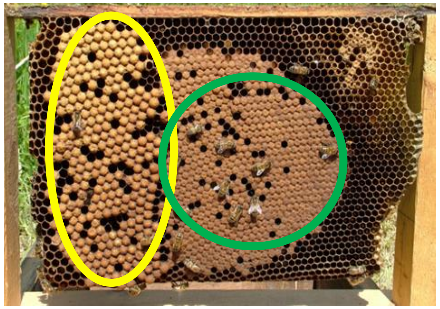
Results: Total amount of comb built

($F_{3,21} = 0.49$, $P = 0.69$)

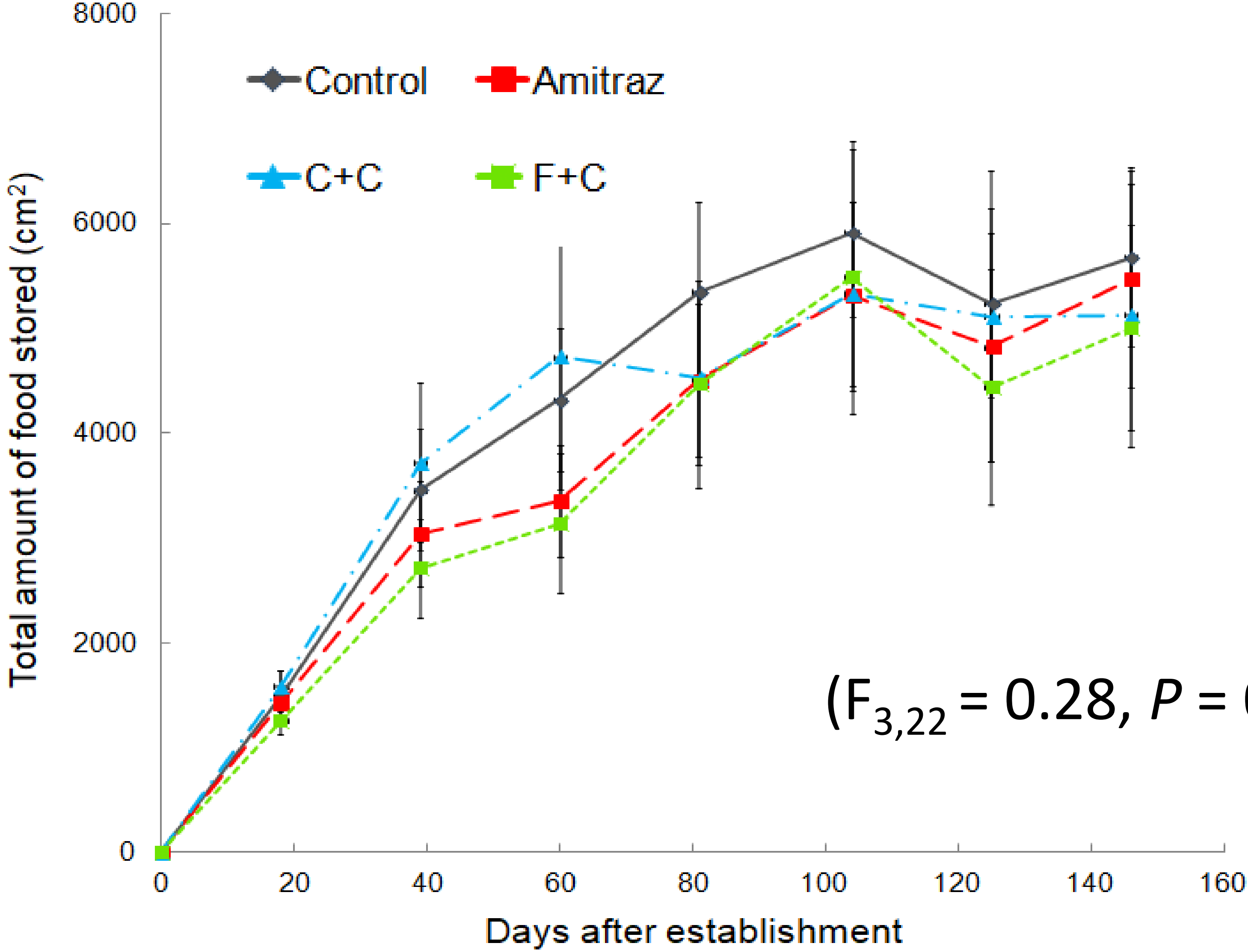


Results: Total amount of sealed brood



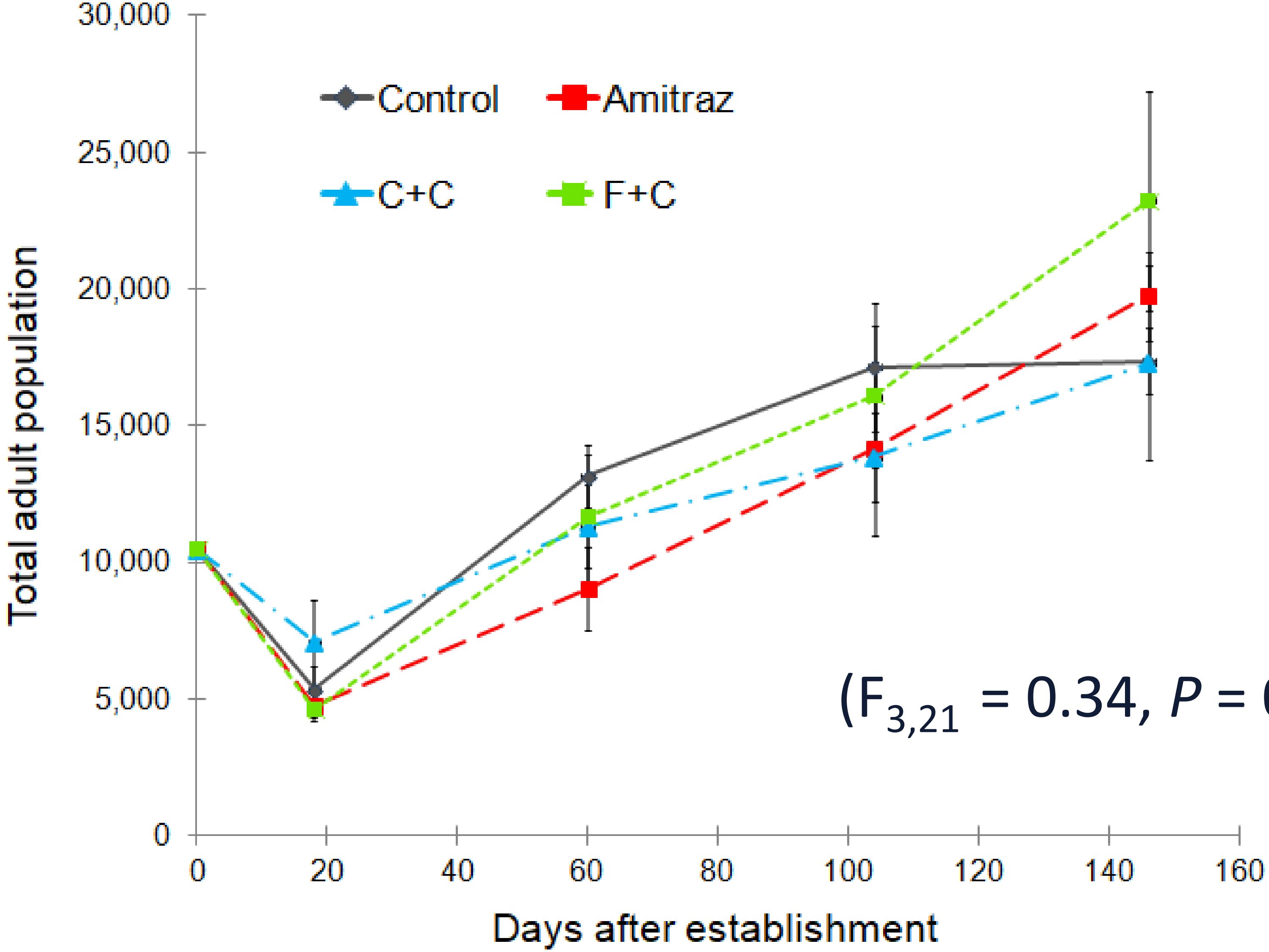


Results: Total amount of food stored



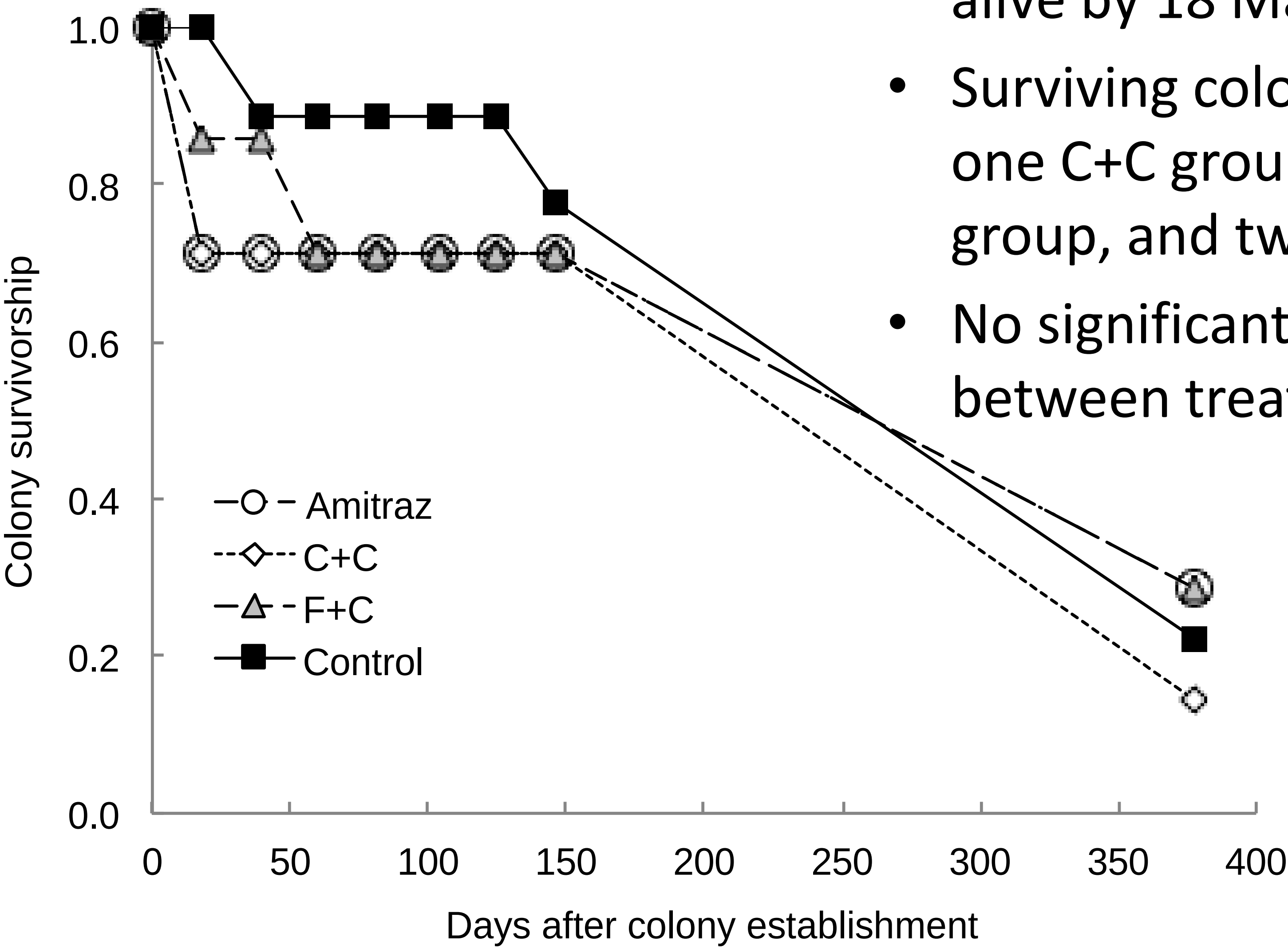
($F_{3,22}$ = 0.28, P = 0.84)

Results: Estimated adult worker population



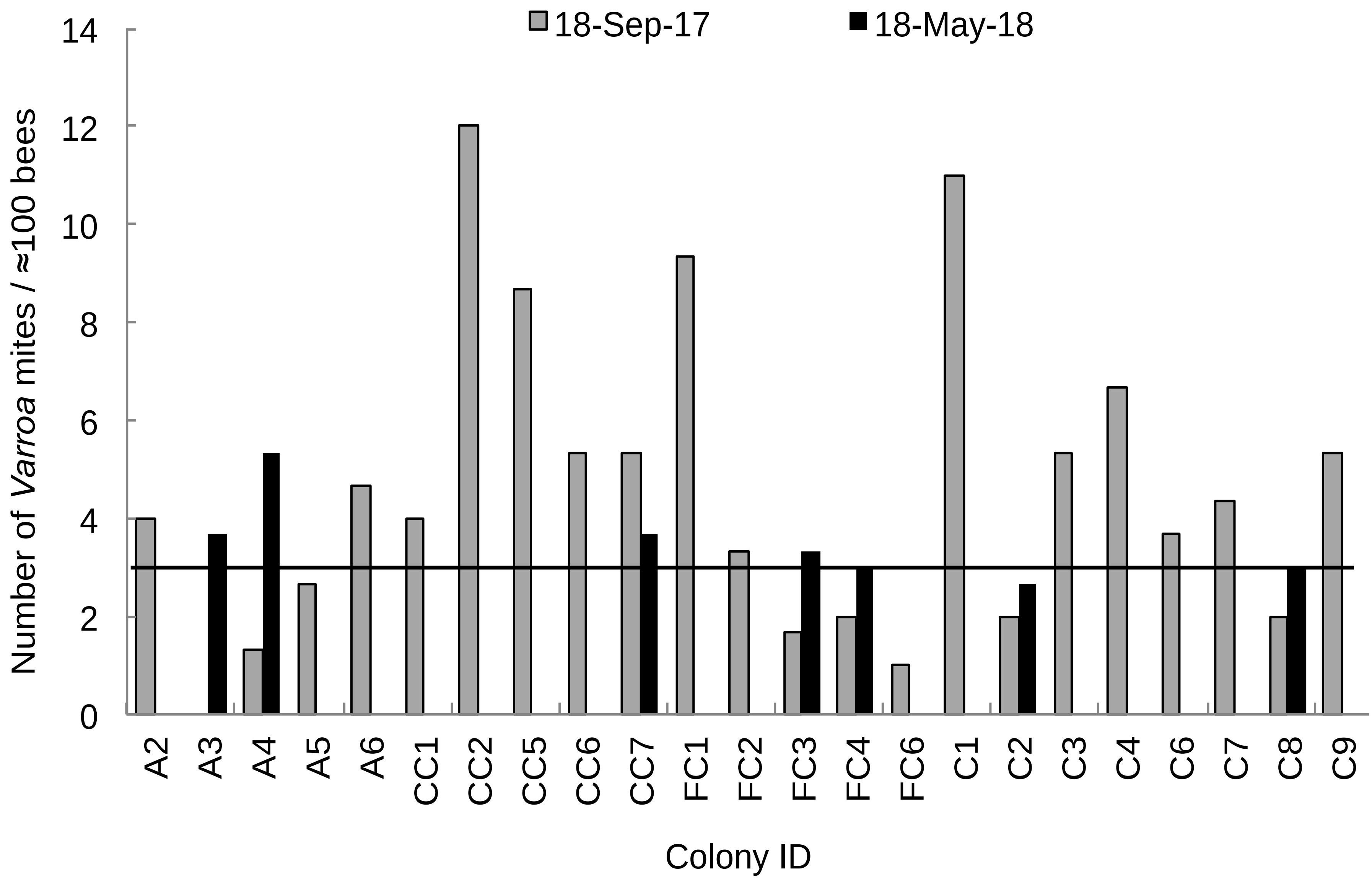
($F_{3,21}$ = 0.34, *P* = 0.79)

Results: Survivorship over winter



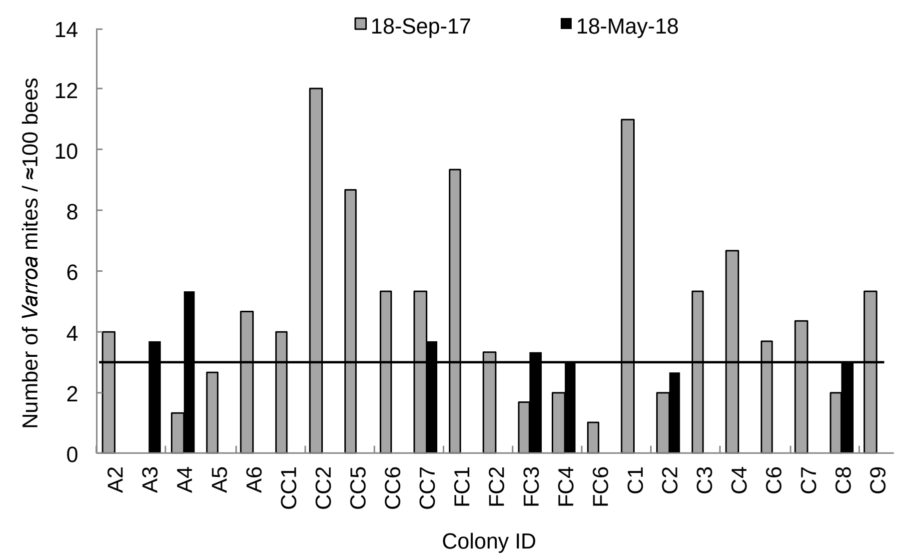
- Only seven of 30 colonies alive by 18 May 2018
- Surviving colonies: two A, one C+C group, two F+C group, and two controls
- No significant differences between treatment groups

Results: *Varroa* levels in surviving colonies



Results: *Varroa* levels in surviving colonies

- Most of the colonies that had a phoretic mite infestation above 3% in Sep. 2017 (65.2%) had died by May 2018
- The colonies that died had significantly more *Varroa* (mean = 5.71 mites / ≈100 bees) than those that had survived (mean = 2.05 mites / ≈100 bees) by May 2018 (t-ratio = 2.96; p = 0.0075) regardless of group
- Six of the seven colonies that survived the full year (85.7%) had a mite level at or below 3% in September 2017, with the exception of one C+C colony that had 5.33 mites per ≈100 bees



Overall conclusions and significance

- For all parameters, the were no statistically significant effects of pesticide contamination in beeswax on overall growth of newly established colonies
- Not whole story… what about burr comb, repeated exposure, old comb??
- Adds to understanding of how pesticides affect honey bee health

QMP differences based on queen quality

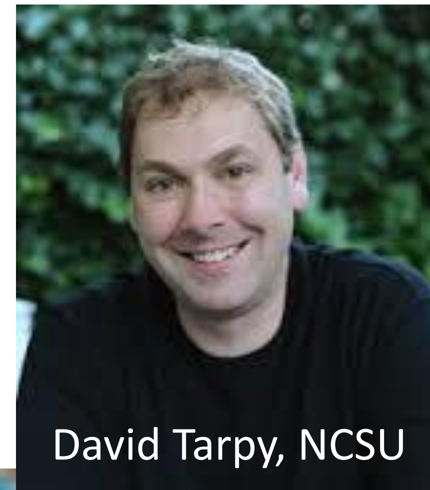
Is the chemical composition of QMP different between queens raised from young vs. old larvae?

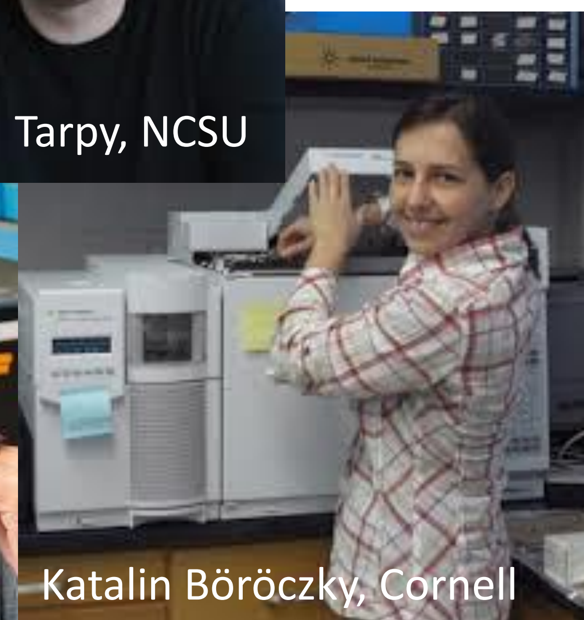
Differential queen rearing

"high" quality queens

"low" quality queens

- Sacrificed the queens and dissected their mandibular glands for chemical analysis of QMP





QMP differences based on queen quality

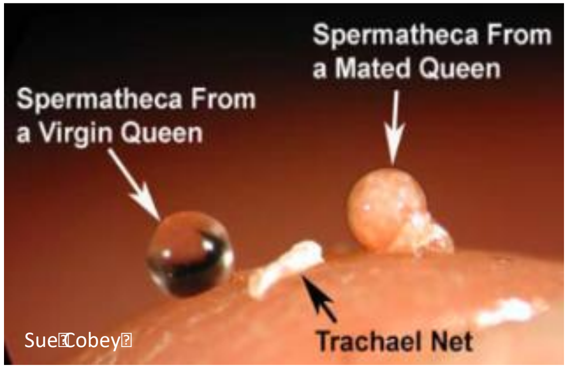
There were significant differences in the relative amounts of HOB, HVA, 9-HDA, 10-HDAA, and 10-HAD (major QMP components) between high- vs. low-quality queens

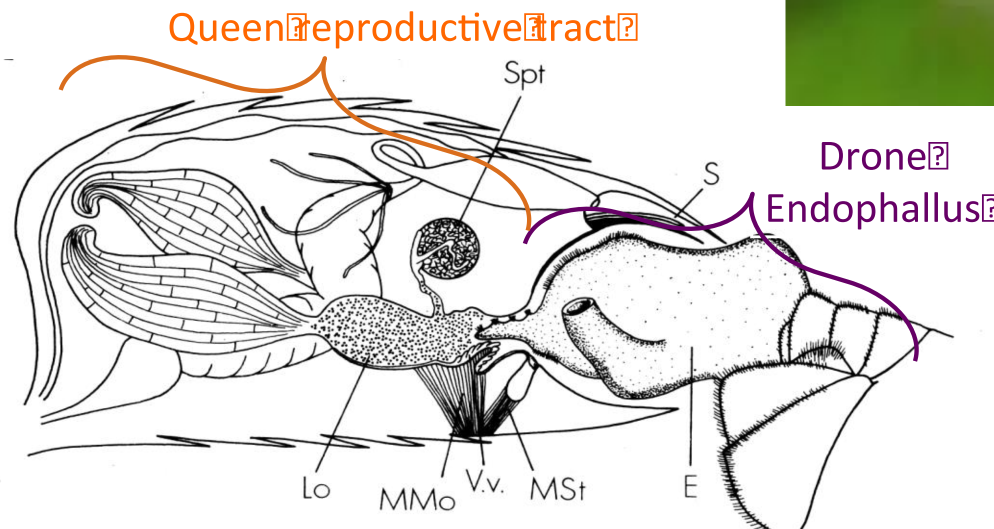
Compound name	QMP code	Kovats Index	Queens raised in 2010							
			High-quality queens			Low-quality queens			Wilcoxon test	
			n	mean	s.e.m.	*n*	mean	s.e.m.	χ^2	*P* value
methyl 4-hydroxybenzoate	**HOB**	1498	9	2.63	0.67	9	4.74	0.67	3.60	**0.05***
unidentified compound 1		1554	9	0.94	0.11	9	0.80	0.11	0.44	0.51
8-hydroxyoctanoic acid	**8-HOAA**	1626	9	8.94	0.88	9	7.96	0.88	0.56	0.45
4-hydroxybenzoic acid		1635	9	0.40	0.05	9	0.39	0.05	0.13	0.72
(*E*)-9-oxo-2-decenoic acid	**9-ODA**	1709	9	39.06	1.37	9	37.44	1.37	0.33	0.57
4-hydroxy-3-methoxyphenylethanol	**HVA**	1720	9	0.27	0.03	9	0.46	0.03	9.59	**0.002**
9-hydroxydecanoic acid		1748	9	1.26	0.09	9	1.16	0.09	1.22	0.27
unidentified compound 2		1783	9	0.55	0.05	9	0.37	0.05	6.61	**0.01**
(*R*)- and (*S*)-(*E*)-9-hydroxy-2-decenoic acid	**9-HDA**	1799	9	28.46	1.13	9	23.34	1.13	7.02	**0.008**
unidentified compound 3		1815	9	0.54	0.05	9	0.47	0.05	1.22	0.27
10-hydroxydecanoic acid	**10-HDAA**	1818	9	1.64	0.33	9	2.44	0.33	5.07	**0.02**
(*E*)-10-hydroxy-2-decenoic acid	**10-HDA**	1869	9	3.48	0.48	9	4.01	0.48	1.32	0.25
4-hydroxy-3-methoxyphenylpropanoic acid		1908	9	0.56	0.04	9	0.67	0.04	3.45	0.06
x-decanedioic acid		1953	9	0.45	0.06	9	0.55	0.06	1.32	0.25
unidentified compound 4		1992	9	0.42	0.02	9	0.40	0.02	0.07	0.79
unidentified compound 5		2020	9	0.62	0.13	9	0.72	0.13	0.28	0.60
x-hexadecenoic acid		2029	9	0.62	0.28	9	1.09	0.28	0.02	0.89
unidentified compound 6		2062	9	0.62	0.11	9	0.84	0.11	0.56	0.45
x-octadecenoic acid		2227	9	0.34	0.15	9	0.63	0.15	0.94	0.33

***Statistically significant differences in mean relative amounts ($P \leq 0.05$) are highlighted in bold**

Importance of queen longevity

- Because of her specialized function in a honey bee colony, the queen's phenotype has been optimized for extreme longevity and reproduction





Implications of our studies

- Little information on mRNAs encoded in sperm from other insect species. But concentrations of certain mRNAs and miRs in sperm are related to the health and viability of sperm in mammals, including humans
- Transcripts encoded in honey bee queen spermathecae and drone semen to identify the effects of stressors on the expression of the genes

Photo: David Tarpy

Photo: Randy Oliver

Mechanisms of long-term sperm storage

- Factors involved in sperm survival inside spermatheca are supplied by spermatheca & semen (den Boer *et al.* 2009)


Nancy Ing

- Uncover key RNAs in sperm in queen spermathecae and drone semen involved in long-term sperm storage
- Generate a gene expression panel of fertility markers in honey bees

RNA-seq of spermathecae and semen

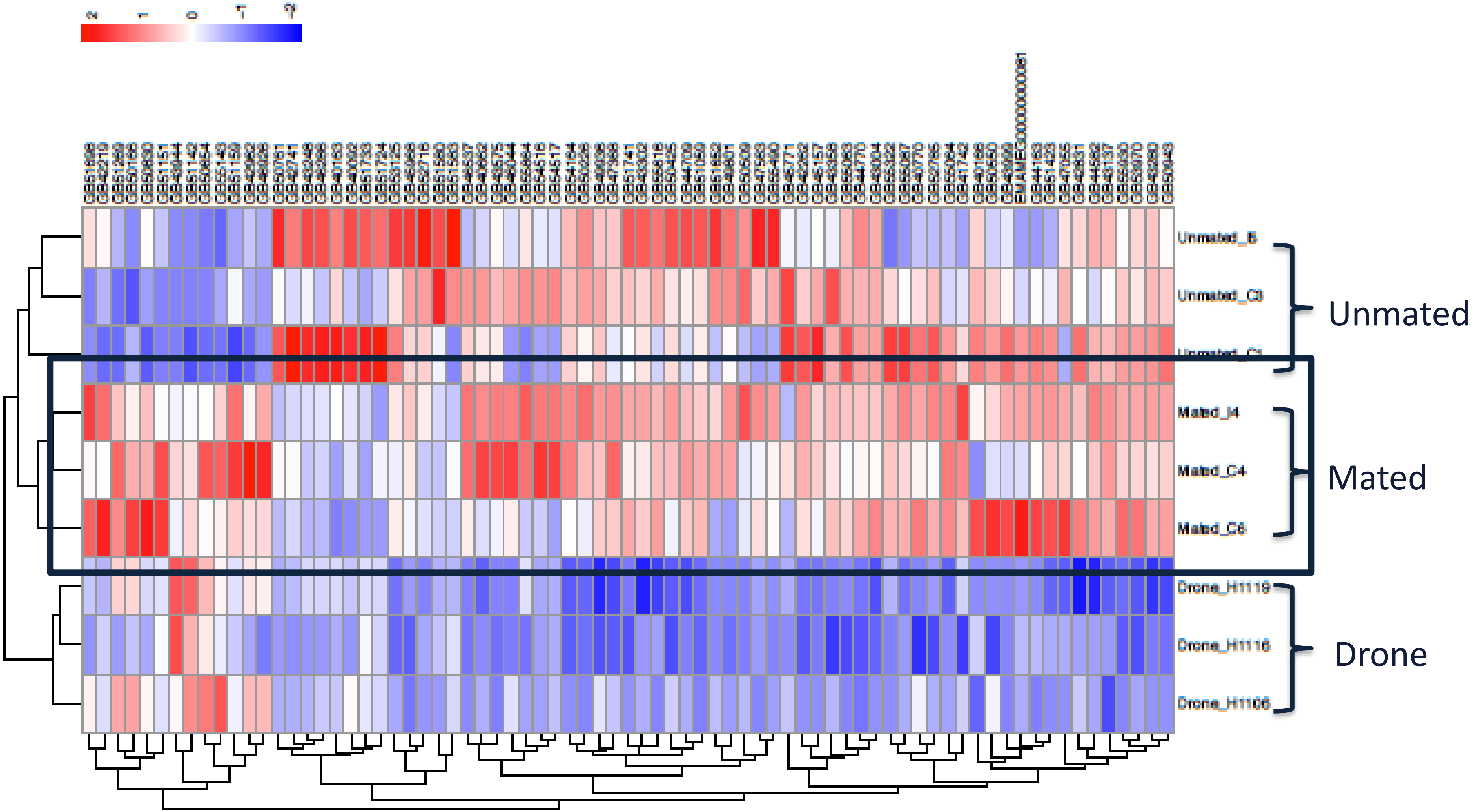
- RNA-sequencing to characterize mRNAs expressed in spermathecae of virgin and mated queens and semen from drones reared naturally

Dr. Alejandra Gonzalez

- cDNA libraries created for:
    - 3 unmated queen spermathecae
    - 3 mated queen spermathecae
    - 3 drone semen ejaculates

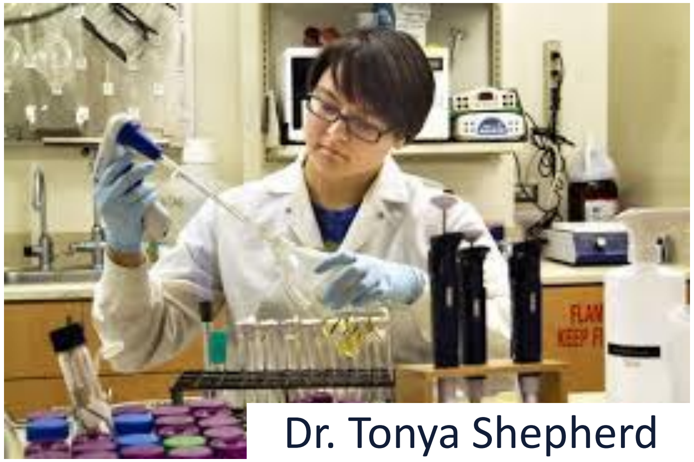
mRNAs in spermathecae and semen

- Identified 10,194 mRNAs across all three tissue types
- 75 most highly expressed mRNAs in spermathecae



Ongoing: RNAs in semen & spermathecae

- Analyzing quantitative RT-PCR results for confirmation of mRNAs more highly expressed in each tissue type
- Sequencing miRs for all tissues
- Characterizing mRNAs and miRs expressed spermathecae of mated vs. virgin queens and in semen from bees reared in pesticide-laden beeswax



Dr. Tonya Shepherd

RNAs in spermathecae and semen

- Quantitative RT-PCR to validate the differential expression of mRNAs most highly expressed in each tissue type
- ef1-alpha (GB41358) used as normalizer (Lourenço *et al.* 2008)

Presumably cleaves the disaccharide trehalose

GB43575	trehalase-like	Mated queens
GB53925	uncharacterized LOC724993	Mated queens
GB54516	uncharacterized LOC100577150	Mated queens
GB43248	alpha glucosidase 2	Unmated queens
GB44112	melittin	Unmated queens
GB54549	alpha-glucosidase	Unmated queens
GB40598	chloride cotransporter	Drone semen
GB48478	multiple inositol polyphosphate phosphatase 1	Drone semen
GB45850	clavesin-2	Drone semen
GB54806	facilitated trehalose transporter Tret1-like	Drone semen

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Gene ID	Gene name from GeneBank	Higher expression in:
	Uncharacterized but increased in seminal fluid by *Nosema* infection	
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GB54516	uncharacterized LOC100577150	Mated queens
GB43248	alpha glucosidase 2	Unmated queens
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	Serine protease abundant in spermatheca (queen-laid egg signal)	
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Cleave glucose from glycoproteins

RNAs in spermathecae and semen

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Known honey bee venom component

RNAs in spermathecae and semen

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Inositol phosphatase enzyme

RNAs in spermathecae and semen

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Involved in endosome/vesicle transport in humans

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Trehalose transporter		
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Honey bee reproductive quality

I. Factors that influence queen and drone reproductive health

I. The effects of commonly used agro-chemicals on queen physiology and worker behavior

I. The effects of commonly used agro-chemicals on drone reproductive health

II. mRNAs in queen spermathecae & drone semen

III. Conclusions and future directions

Implications of our studies

- Little information on mRNAs encoded in sperm from other insect species. But concentrations of certain mRNAs and miRs in sperm are related to the health and viability of sperm in mammals, including humans
- Transcripts encoded in honey bee queen spermathecae and drone semen to identify the effects of stressors on the expression of the genes

Photo: David Tarpy

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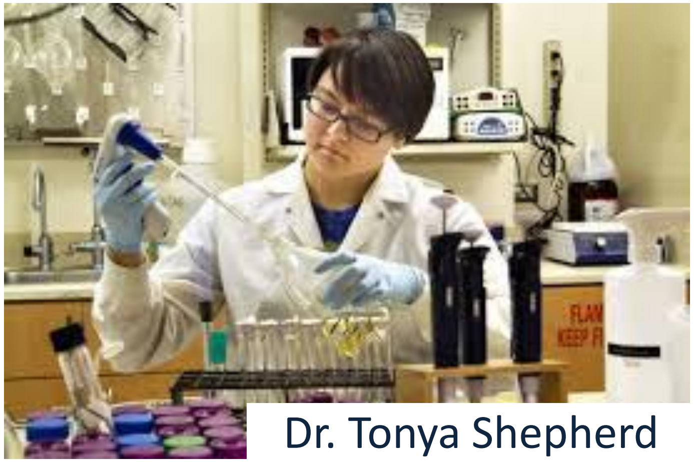
- Transcripts encoded in honey bee queen spermathecae and drone semen to identify the effects of stressors on the expression of the genes
- We hope to identify a “fingerprint” of gene expression that could aid in predicting queen reproductive quality
- Ultimately our goal is to unravel the contribution of key genes to sperm survivorship and viability

Ongoing: RNAs in semen & spermathecae

- Analyzing quantitative RT-PCR results for confirmation of mRNAs more highly expressed in each tissue type
- Sequencing miRs for all tissues
- Characterizing mRNAs and miRs expressed spermathecae of mated vs. virgin queens and in semen from bees reared in pesticide-laden beeswax



Dr. Tonya Shepherd